National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

UNIVERSITY OF ALBERTA

CAPILLARY GEL ELECTROPHORESIS FOR DNA SEQUENCING

BY

HEATHER R. STARKE

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

DEPARTMENT OF CHEMISTRY

Edmonton, Alberta
FALL 1994

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-95270-9

Canada

Name HEATHER STARKE

Dissertation Abstracts International is arranged by broad, general subject categories. Please select the one subject which most nearly describes the content of your dissertation. Enter the corresponding four-digit code in the spaces provided.

Analytical Chemistry
SUBJECT TERM

0486
SUBJECT CODE

U·M·I

Subject Categories

THE HUMANITIES AND SOCIAL SCIENCES

COMMUNICATIONS AND THE ARTS
- Architecture 0729
- Art History 0377
- Cinema 0900
- Dance 0378
- Fine Arts 0357
- Information Science 0723
- Journalism 0391
- Library Science 0399
- Mass Communications 0708
- Music 0413
- Speech Communication 0459
- Theater 0465

EDUCATION
- General 0515
- Administration 0514
- Adult and Continuing 0516
- Agricultural 0517
- Art 0273
- Bilingual and Multicultural 0282
- Business 0688
- Community College 0275
- Curriculum and Instruction 0727
- Early Childhood 0518
- Elementary 0524
- Finance 0277
- Guidance and Counseling 0519
- Health 0680
- Higher 0745
- History of 0520
- Home Economics 0278
- Industrial 0521
- Language and Literature 0279
- Mathematics 0280
- Music 0522
- Philosophy of 0998
- Physical 0523
- Psychology 0525
- Reading 0535
- Religious 0527
- Sciences 0714
- Secondary 0533
- Social Sciences 0534
- Sociology of 0340
- Special 0529
- Teacher Training 0530
- Technology 0710
- Tests and Measurements 0288
- Vocational 0747

LANGUAGE, LITERATURE AND LINGUISTICS
- Language
  - General 0679
  - Ancient 0289
  - Linguistics 0290
  - Modern 0291
- Literature
  - General 0401
  - Classical 0294
  - Comparative 0295
  - Medieval 0297
  - Modern 0298
  - African 0316
  - American 0591
  - Asian 0305
  - Canadian (English) 0352
  - Canadian (French) 0355
  - English 0593
  - Germanic 0311
  - Latin American 0312
  - Middle Eastern 0315
  - Romance 0313
  - Slavic and East European 0314

PHILOSOPHY, RELIGION AND THEOLOGY
- Philosophy 0422
- Religion
  - General 0318
  - Biblical Studies 0321
  - Clergy 0319
  - History of 0320
  - Philosophy of 0322
- Theology 0469

SOCIAL SCIENCES
- American Studies 0323
- Anthropology
  - Archaeology 0324
  - Cultural 0326
  - Physical 0327
- Business Administration
  - General 0310
  - Accounting 0272
  - Banking 0770
  - Management 0454
  - Marketing 0338
- Canadian Studies 0385
- Economics
  - General 0501
  - Agricultural 0503
  - Commerce-Business 0505
  - Finance 0508
  - History 0509
  - Labor 0510
  - Theory 0511
- Folklore 0358
- Geography 0366
- Gerontology 0351
- History
  - General 0578
  - Ancient 0579
  - Medieval 0581
  - Modern 0582
  - Black 0328
  - African 0331
  - Asia, Australia and Oceania 0332
  - Canadian 0334
  - European 0335
  - Latin American 0336
  - Middle Eastern 0333
  - United States 0337
- History of Science 0585
- Law 0398
- Political Science
  - General 0615
  - International Law and Relations 0616
  - Public Administration 0617
- Recreation 0814
- Social Work 0452
- Sociology
  - General 0626
  - Criminology and Penology 0627
  - Demography 0938
  - Ethnic and Racial Studies 0631
  - Individual and Family Studies 0628
  - Industrial and Labor Relations 0629
  - Public and Social Welfare 0630
  - Social Structure and Development 0700
  - Theory and Methods 0344
- Transportation 0709
- Urban and Regional Planning 0999
- Women's Studies 0453

THE SCIENCES AND ENGINEERING

BIOLOGICAL SCIENCES
- Agriculture
  - General 0473
  - Agronomy 0285
  - Animal Culture and Nutrition 0475
  - Animal Pathology 0476
  - Food Science and Technology 0359
  - Forestry and Wildlife 0478
  - Plant Culture 0479
  - Plant Pathology 0480
  - Plant Physiology 0817
  - Range Management 0777
  - Wood Technology 0746
- Biology
  - General 0306
  - Anatomy 0287
  - Biostatistics 0308
  - Botany 0309
  - Cell 0379
  - Ecology 0329
  - Entomology 0353
  - Genetics 0369
  - Limnology 0793
  - Microbiology 0410
  - Molecular 0307
  - Neuroscience 0317
  - Oceanography 0416
  - Physiology 0433
  - Radiation 0821
  - Veterinary Science 0778
  - Zoology 0472
- Biophysics
  - General 0786
  - Medical 0760

EARTH SCIENCES
- Biogeochemistry 0425
- Geochemistry 0996
- Geodesy 0370
- Geology 0372
- Geophysics 0373
- Hydrology 0388
- Mineralogy 0411
- Paleobotany 0345
- Paleoecology 0426
- Paleontology 0418
- Paleozoology 0985
- Palynology 0427
- Physical Geography 0368
- Physical Oceanography 0415

HEALTH AND ENVIRONMENTAL SCIENCES
- Environmental Sciences 0768
- Health Sciences
  - General 0566
  - Audiology 0300
  - Chemotherapy 0992
  - Dentistry 0567
  - Education 0350
  - Hospital Management 0769
  - Human Development 0758
  - Immunology 0982
  - Medicine and Surgery 0564
  - Mental Health 0347
  - Nursing 0569
  - Nutrition 0570
  - Obstetrics and Gynecology 0380
  - Occupational Health and Therapy 0354
  - Ophthalmology 0381
  - Pathology 0571
  - Pharmacology 0419
  - Pharmacy 0572
  - Physical Therapy 0382
  - Public Health 0573
  - Radiology 0574
  - Recreation 0575
  - Speech Pathology 0460
  - Toxicology 0383
- Home Economics 0386

PHYSICAL SCIENCES

Pure Sciences
- Chemistry
  - General 0485
  - Agricultural 0749
  - Analytical 0486
  - Biochemistry 0487
  - Inorganic 0488
  - Nuclear 0738
  - Organic 0490
  - Pharmaceutical 0491
  - Physical 0494
  - Polymer 0495
  - Radiation 0754
- Mathematics 0405
- Physics
  - General 0605
  - Acoustics 0986
  - Astronomy and Astrophysics 0606
  - Atmospheric Science 0608
  - Atomic 0748
  - Electronics and Electricity 0607
  - Elementary Particles and High Energy 0798
  - Fluid and Plasma 0759
  - Molecular 0609
  - Nuclear 0610
  - Optics 0752
  - Radiation 0756
  - Solid State 0611
- Statistics 0463

Applied Sciences
- Applied Mechanics 0346
- Computer Science 0984
- Engineering
  - General 0537
  - Aerospace 0538
  - Agricultural 0539
  - Automotive 0540
  - Biomedical 0541
  - Chemical 0542
  - Civil 0543
  - Electronics and Electrical 0544
  - Heat and Thermodynamics 0348
  - Hydraulic 0545
  - Industrial 0546
  - Marine 0547
  - Materials Science 0794
  - Mechanical 0548
  - Metallurgy 0743
  - Mining 0551
  - Nuclear 0552
  - Packaging 0549
  - Petroleum 0765
  - Sanitary and Municipal 0554
  - System Science 0790
- Geotechnology 0428
- Operations Research 0796
- Plastics Technology 0795
- Textile Technology 0994

PSYCHOLOGY
- General 0621
- Behavioral 0384
- Clinical 0622
- Developmental 0620
- Experimental 0623
- Industrial 0624
- Personality 0625
- Physiological 0989
- Psychobiology 0349
- Psychometrics 0632
- Social 0451

The undersigned grant permission that the material published in

Journal of Chromatography, 1992, 608, 143-150

entitled: "Effect of total percent polyacrylamide in capillary gel electrophoresis for DNA sequencing of short fragments: A phenomenological model"

be used in the Ph.D. thesis of Heather R. Starke

Sue Bay

Jian Zhong Zhang

Marie Josée Rocheleau

Norman J. Dovichi

The undersigned grant permission tha the material published in

Nucleic Acids Research, 1992, 20, 4873-4880

entitled: "Two-label peak-height encoded DNA sequencing by capillary gel electrophoresis: three examples"

be used in the Ph.D. thesis of Heather R. Starke

DaYong Chen

Norman J. Dovichi

The undersigned grant permission that the material published in

Advances in DNA Sequencing, SPIE, Vol. 1891 (1993), p8.

entitled: "Accuracy of two-color peak height encoded DNA sequencing by capillary gel electrophoresis and laser-induced fluorescence"

be used in the Ph.D. thesis of Heather R. Starke

Sue Bay

John Elliott

Norman Dovichi

The undersigned grant permission that the material submitted for publication in

Nucleic Acids Research

entitled: "Internal fluorescence labeling with fluorescent deoxynucleotides in two-label peak-height encoded DNA sequencing by capillary electrophoresis"

be used in the Ph.D. thesis of Heather R. Starke

Yan, Juying

Ju Ying Yan

J. Zhang

Jian Zhong Zhang

Klaus Mühlegger

Klaus Effgen

Norman J. Dovichi

UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR:	Heather R. Starke
TITLE OF THESIS:	Capillary Gel Electrophoresis for DNA Sequencing
DEGREE:	Doctor of Philosophy
YEAR THIS DEGREE GRANTED:	1994

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as hereinbefore provided neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material from whatever without the author's prior written permission .

Heather Starke

9830 - 72 Ave
Edmonton, Alberta, T6E 0Z1

Date: 6 October 1994

UNIVERSITY OF ALBERTA
FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Capillary Gel Electrophoresis for DNA Sequencing** submitted by **Heather R. Starke** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**.

Dr. N.J. Dovichi

Dr. F.F. Cantwell

Dr. J.E. Bertie

Dr. B.G. Kratochvil

Dr. J.F. Elliott

Dr. B. Koop

Abstract

The advent of large scale DNA sequencing projects drives the continued development of sequencing technologies. Capillary gel electrophoresis (CGE), coupled with a highly sensitive laser induced fluorescence detector, is a high speed technique that is used to separate fluorescently tagged DNA sequencing samples. The behaviour of short fragments (less than 250 bases) is described as a function of total percent acrylamide (%T) in gels with constant crosslinker concentration (5%C). At each concentration peak spacing was constant. The peak spacing increased linearly with the total percent acrylamide. Theoretical plate counts are limited by longitudinal diffusion, and are independent of total percent acrylamide.

A two-label peak-height encoded sequencing technique that is a variation on the peak-height method of Tabor and Richardson was demonstrated. The samples generated with the ABI FAM and JOE labelled primers were readily detected on the CGE instrument constructed for use with the DuPont Genesis sequencing system. A peak height ratio of 2:1 gave sequencing accuracies of 98%, while a peak height ratio of 3:1 gave accuracies of more than 99%.

The performance of the CGE using the two-colour peak-height ratio method was evaluated. Sequence data was generated for five samples of malaria DNA on the CGE instrument and the Pharmacia ALF sequencer. The sequence data was compared to the consensus sequence. The length of the sequence data obtained per run ranged from 300-460, and the accuracy of the sequences obtained by CGE ranged from 97.3-99.8%. The lengths and accuracies were comparable to those from the automated sequencer. The CGE instrument produces the data about 3 times faster than the Pharmacia instrument.

Internal labelling of the sequencing samples was accomplished with fluorescently tagged dUTP and dATP. Fluorescein and tetramethylrhodamine labelled dATPs were used to generate two-colour peak-height encoded samples. The detector was modified to detect these two labels: two lasers, a green HeNe and an Argon ion laser were used to excite the tetramethylrhodamine and fluorescein respectively. Internal labelling provides an simple, inexpensive alternative to primer labelling.

Acknowledgements

Capillary electrophoresis experiments are not carried out in a vacuum. Many people contributed in many ways to the work described in this thesis.

First and foremost, I would like to express my sincere appreciation to Dr. Norman "hands of a sturgeon" Dovichi. From first to last he has been a real encouragement and help. I have learned a great deal from him during the last five years and his gift for choosing research topics is reflected in this thesis.

I would also like to mention all of the past and present members of the Northern Lights Laser Lab. Most of them contributed in some way to this project specifically, and to my overall education. We have shared a lot of experiences and bad puns over the years, we have experienced growing pains as the group expanded, and we have become good friends as well as colleagues. Specifically, I owe a great deal to Dr. Harold Swerdlow, who started the DNA research and guided us through the early work. Dr. David Chen bailed me out of countless problems in the lab. I have always appreciated his quiet patience and encouragement. Sue Bay, summer student extraordinaire, was responsible for a great deal of the work presented in chapters 2 and 4. It was a joy to work with Sue.

The excellent support staff in the machine shop, the electronics shop, and the offices also deserve mention. Without them to keep things running smoothly this work would have taken much longer.

My sincere thanks to Dr. John Elliott and Dean Smith of the Departments of Medical Microbiology and Infections Diseases and Immunology, for hands on training in the fine art of sequencing. One slab gel was enough to convince me that capillaries held a lot of promise for the field. Dr. Satyender Hansra provided us with the malaria clones as well as her sequence data for the comparison in Chapter 4.

Dr. Carl Fuller of United States Biochemical Corporation also contributed immensely to this work, providing reagents and enzymes, technical expertise and many entertaining telephone conversations.

Finally, I would like to thank my parents, Siegfried and Margaret Harke, and my husband Dieter Starke for their unfailing love and encouragement.

Table of Contents

CHAPTER 1 Introduction 1

1.1 The Structure of DNA 1

1.2 DNA Sequencing Techniques 5

1.2.1 Chain Extension and Termination - Sanger's Method 6

1.2.2 The Maxam-Gilbert Chemical Degradation Technique 8

1.3 Modified Sequencing Approaches 13

1.3.1 One Fluorescent Primer 13

1.3.2 Four Fluorescent Primers 14

1.3.3 Dye Terminators 14

1.3.4 Internal Labelling with Fluorescent dNTPs 15

1.3.5 Peak Height Methods 16

1.3.6 Cycle Sequencing 16

1.4 Mapping and Sequencing the Genome 18

1.4.1 Restriction Digests 18

1.4.2 Gel Electrophoresis 20

1.4.3 Cloning 21

1.4.4 Mapping and Sequencing 21

1.5 Capillary Electrophoresis 23

1.6 Laser-Induced Fluorescence 26

References 32

CHAPTER 2 The Effect of Total Percent Polyacrylamide in Capillary Gel Electrophoresis for DNA Sequencing of Short Fragments 35

2.1 Introduction 35

2.2 Experimental 36

2.2 Experimental 36
2.2.1 Instrument Design 36
2.2.2 Gel Preparation 40
2.2.3 Sample Preparation 41
2.2.4 Electrophoresis 41
2.3 Results and Discussion 42
2.3.1 Performance and Stability of the Gel Filled Capillaries 42
2.3.2 Sequencing Rate and Retention Time 47
2.3.3 Electrophoretic Mobility 51
2.3.4 Theoretical Plates 55
2.3.5 Band Broadening - injection, detection, and thermal gradient 55
2.3.6 Band Broadening - longitudinal diffusion 58
2.3.7 Resolution 60
2.4 Conclusions 63
References 65
CHAPTER 3 Two-Label Peak Height Encoded DNA Sequencing by Capillary Gel Electrophoresis 67
3.1 Introduction 67
3.2 Experimental 69
3.2.1 Electrophoresis 69
3.2.2 Detector 69
3.2.3 Sample Preparation 71
3.2.4 Sequence Determination 72
3.3 Results and Discussion 72
3.4 Conclusions 94

References 96

CHAPTER 4 Accuracy of Two-Colour Peak Height Encoded DNA Sequencing by Capillary Gel Electrophoresis and Laser-Induced Fluorescence 97

4.1 Introduction 97

4.2 Experimental 99

4.2.1 Instrument Design 99

4.2.2 Gel Preparation 99

4.2.3 Sample Preparation 100

4.2.4 Electrophoresis 101

4.2.5 ALF Sequencing 102

4.3 Results and Discussion 102

4.3.1 Sequence Accuracy 103

4.3.2 Sequence Length 119

4.3.3 Sequencing Rate 120

4.3.4 Resolution 120

4.4 Conclusions 121

References 123

CHAPTER 5 Internal Fluorescence Labelling with Two-Label Peak-Height Encoded DNA Sequencing by Capillary Gel Electrophoresis 124

5.1 Introduction 124

5.2 Experimental 127

5.2.1 Instrument Design 127

5.2.2 Detector 128

5.2.3 Sample Preparation 128

5.2.4 Electrophoresis 132

5.3 Results and Discussion 132

5.3.1 F-dUTP Labelled Sample 132
5.3.2 Two-Colour dATP Labelled Sample 133
5.3.3 The Labelling Reaction 137
5.3.4 Comparison of Separation of Internal and Primer Labelled Samples 146
5.4 Conclusions 155
References 156
CHAPTER 6 Conclusions and Future Work 157
6.1 Conclusions 157
6.2 Future Work 158
References 160
Appendix A Consensus Sequences 161
1. M13mp18 161
2. Mouse mammary tumour virus - MMTV 164
3. Mouse cytokine - 123 delta 165
4. Malaria templates 1 to 5 166
Appendix B Problems in DNA Sequencing 171
1. Compressions 171
2. Non-specific Priming 173
3. Degraded Template DNA 176

List of Tables

Table 1.1 Chemical Modifications for Maxam-Gilbert Sequencing..... 10

Table 1.2 Some restriction enzymes and their recognition sites..... 19

Table 4.1 Length and accuracy of DNA sequence determined by CGE and automated slab gel electrophoresis..... 118

List of Figures

Figure 1.1 2

a. A Nucleotide: the basic repeating unit of DNA 2

b. The purine and pyrimidine bases 2

Figure 1.2 Single stranded DNA 3

Figure 1.3 Watson-Crick base pairs 4

Figure 1.4 The chain extension and termination reactions 7

Figure 1.5 Slab gel electrophoresis of the chain termination reaction products 9

Figure 1.6 The Maxam-Gilbert chemical degradation reactions 11

Figure 1.7 Slab gel electrophoresis of a Maxam-Gilbert sequencing sample 12

Figure 1.8 The peak height method developed by Richardson and Tabor 17

Figure 1.9 19

a. Polymerase chain reaction 19

b. Cycle sequencing 19

Figure 1.10 25

a. Random or shotgun sequencing 25

b. Primer walking 25

Figure 1.11 The succinylfluorescein dyes used by DuPont 30

Figure 1.12 The ABI fluorescent dyes 31

Figure 2.1 37

a. Schematic diagram of the capillary electrophoresis instrument 37

b. The sheath flow cuvette 38

Figure 2.2 Schematic diagram of the one-laser two-channel fluorescence detector 39

Figure 2.3 Electropherogram of an A-terminated M13mp18 sequencing sample 44

Figure 2.4 Retention time as a function of fragment length for single stranded DNA sequencing fragments 45
Figure 2.5 Peak spacing of DNA fragments for denaturing polyacrylamide gels 46
Figure 2.6 Sequencing rate for the data of Figure 2.4 48
Figure 2.7 Retention time for a vanishingly small DNA fragment as a function of total acrylamide concentration 49
Figure 2.8 Mobility of fluorescently labelled DNA fragments 52
Figure 2.9 Plate count for an 85 base DNA sequencing fragment as a function of total acrylamide concentration 54
Figure 2.10 Predicted plate count in a 4% total acrylamide gel 59
Figure 2.11 Resolution of bases 85-86 61
Figure 2.12 Predicted resolution for adjacent DNA fragments as a function of total acrylamide concentration 62
a. Frements 250-251 bases in length 62
b. Fragments 500-501 bases in length 62
Figure 3.1 Diagram of the two-channel fluorescence detector 70
Figure 3.2 76
a. Electropherogram of A and C terminated MMTV DNA sequencing sample 76
b. The expanded electropherogram 78
c. Fragments 40 to 50 and 320 to 330 nucleotides in length 79
Figure 3.3 81
a. Electropherogram of the T and G terminated MMTV DNA sequencing sample 81
b. The expanded electropherogram 83
c. Fragments 20 to 30 and 110 to 120 nucleotides in length 84

Figure 3.4 Electropherogram of MMTV sample with a peak height ratio of 2:1 86

Figure 3.5 Electropherogram of an MMTV sample with a peak height ratio of 3:1 89

Figure 3.6 Electropherogram of an M13mp18 sample 91

Figure 3.7 Electropherogram of mouse cytokine DNA sample 93

Figure 4.1 105

a. Electropherogram of malaria DNA clone 1 105

b. Expansion showing fragments 100 to 150 and 350 to 400 nucleotides 107

Figure 4.2 109

a. Electropherogram of malaria DNA clone 2 109

b. Electropherogram of malaria DNA clone 2 - different peak height coding 111

Figure 4.3 Electropherogram of malaria DNA clone 3 113

Figure 4.4 Electropherogram of malaria DNA clone 4 115

Figure 4.5 Electropherogram of malaria DNA clone 5 117

Figure 4.6 Comparison of resolution in automated slab gel electrophoresis and CGE 122

Figure 5.1 The bases thymine and uracil 125

Figure 5.2 Diagram of the two-laser, two-channel fluorescence detector 129

Figure 5.3 Electropherogram of an A and C teminated M13mp18 sample labelled with F-12-dUTP 135

Figure 5.4 Sequencing Electropherogram for the two-colour internally labelled M13mp18 sample 139

Figure 5.5 Data processing to compensate for the mobility shift 140

Figure 5.6 Electropherogram of the internally labelled MMTV sample 142

Figure 5.7 Electropherogram of the internally labelled malaria sample 144
Figure 5.8 Electropherogram of the products of the labelling reaction 145
Figure 5.9 148
a. Electropherogram of the internally labelled C terminated M13mp18 sample 148
b. Electropherogram of the primer labelled C terminated M13mp18 sample 150
Figure 5.10 Retention time vs fragment length for internal and primer labelled samples 151
Figure 5.11 153
a. Inverse fragment length vs. mobility 153
b. A straight line fit to the data for fragments longer than 200 bases 153
Figure 5.12 Resolution as a function of fragment length for primer and internal labelled samples 154
Figure B.1 The effect of formamide on compressions 172
Figure B.2 Non-specific priming 175
Figure B.3 Template degradation 178
a. A C-terminated sample 178
b. An A-terminated sample 180

CHAPTER 1

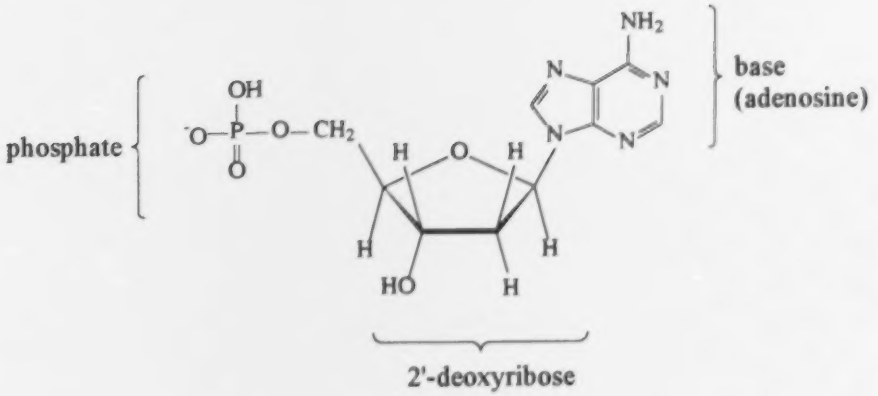
Introduction

Deoxyribonucleic acid (DNA) has been dubbed the blueprint for life. It is one of the most highly studied molecules, fascinating scientists for four decades. DNA was once described by Maurice Wilkins as "...Midas' gold. Everyone who touches it goes mad..." (1). DNA continues to fascinate. A current quest of scientists and organisations all over the world is the sequence of the DNA which makes up the human genome. On a smaller scale, the sequences of various genes are also sought. The very scale of such sequencing projects drives the continued development of new technologies. The subject of this thesis is the application of capillary gel electrophoresis with laser induced fluorescence detection to DNA sequencing.

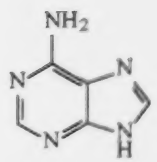
1.1 The Structure of DNA

DNA was first identified as the genetic principle in 1944 by Oswald Avery (2). By the year 1953, Watson and Crick had elucidated the structure of DNA (3). DNA is a long, straight chain polymer. The basic repeating unit of DNA is the nucleotide (Figure 1.1a). A nucleotide is composed of three substituents: 2'-deoxyribose, a phosphate group, and a purine or pyrimidine base. There are four different bases which may be attached to the ribose ring (Figure 1.1b), adenosine (A) and guanine (G), cytosine (C) and thymine (T) . The nucleotides are joined together by a phosphodiester bond to form DNA. The 3' hydroxyl group of one nucleotide is joined to the 5' hydroxyl of the next. A strand of DNA has polarity: there is a 5' end with a free phosphate group, and 3' end with a free hydroxyl group. Single stranded DNA is shown in figure 1.2.

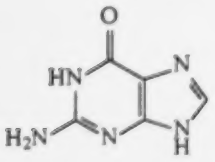
a.



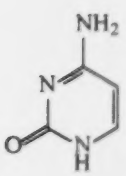
b.



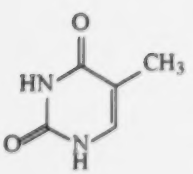
Adenosine



Guanine



Cytosine



Thymine

Figure 1.1

a. A nucleotide (deoxyadenosine monophosphate) is the basic repeating unit of DNA. The nucleotide is composed of three parts, a purine or pyrimidine base, in this case adenine, a 2'-deoxyribose, and a phosphate group.

b. The purine bases (top row), and the pyrimidine bases (bottom row)

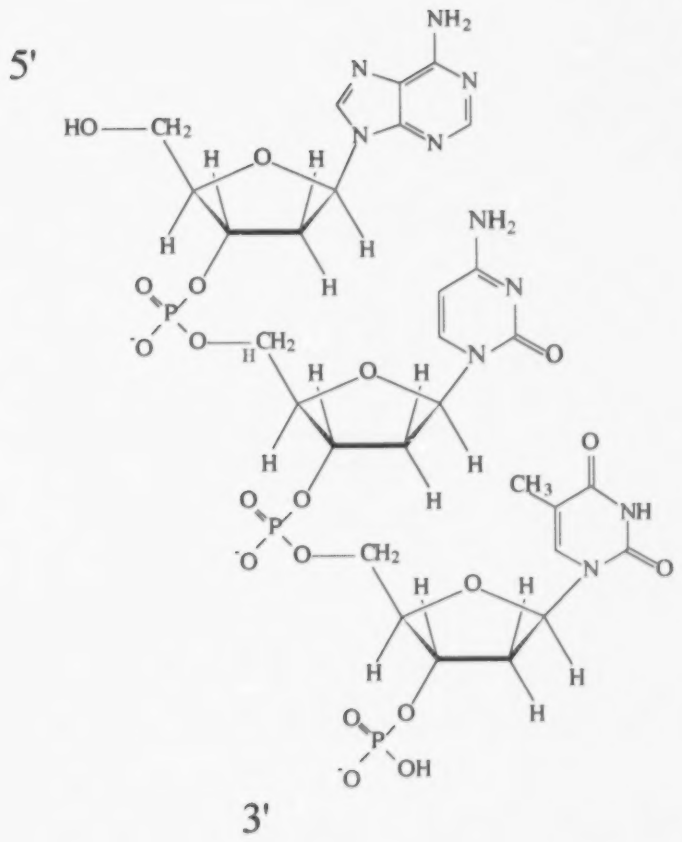


Figure 1.2 Single stranded DNA. This trinucleotide may be represented using the one letter codes for the bases as 5' ACT 3'.

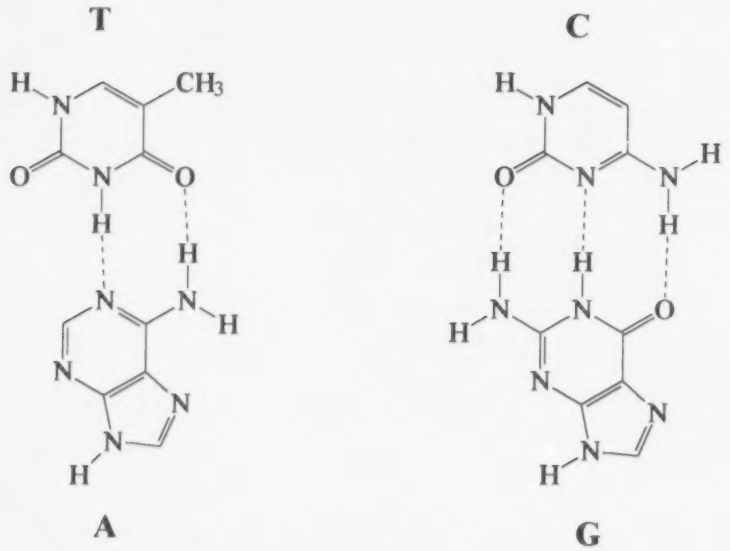


Figure 1.3 Watson-Crick base pairs. Adenine (A) pairs with thymine (T), on the left, and guanine(G) pairs with cytosine (C), on the right.

Generally DNA exists in the double stranded form, *i.e.* two DNA strands are associated. The two strands are held together by hydrogen bonds that form between the bases. The position of the hydrogen bonds makes pairing quite exclusive; A pairs with T and G pairs with C to form the Watson-Crick base pairs shown in Figure 1.3. The two strands of DNA are complementary by virtue of the base pairing: where there is A in one strand there is T in the other, where there is G in one strand there is C in the other. The two strands are also antiparallel; the 3' end of one strand lines up with the 5' end of the other strand.

The hydrogen bonds that hold the two strands together may be broken by raising the pH or the temperature of the DNA. In this way the DNA is denatured. Lowering pH or temperature means that the hydrogen bonds reform, *i.e.* the strands anneal. Short strands of DNA (oligonucleotides) which are complementary to a stretch of the DNA also easily anneal or hybridise.

1.2 DNA Sequencing Techniques

The sequence of DNA is the order of the nucleotides in a particular strand of DNA. The variable part of the DNA is the base attached to the sugar-phosphate backbone. The basic principle of most DNA sequencing techniques is the generation of a set of DNA fragments, which have one end in common and which differ in length by one nucleotide. In this way the sequence of nucleotides is converted into chain length information. What is needed to sequence the nucleotides is: 1) a way to generate the fragments, 2) a way to separate them on the basis of size, and 3) a way to determine the identity of the unique end of each fragment.

The most widely used method which generates a nested set of DNA fragments is the chain extension and termination method developed by Sanger (4). A second method based on chemical degradation of the DNA was developed by Maxam and Gilbert (5).

1.2.1 Chain Extension and Termination - Sanger's method

The chain extension and termination method developed by Sanger (4) makes use of the enzyme DNA polymerase. This enzyme requires a template DNA molecule of interest and a short oligonucleotide primer that is complementary to a known portion of the template. The polymerase sythesises a strand of DNA that is complementary to the template DNA by successive addition of the appropriate deoxynucleotide to the 3' hydroxyl group of the primer. The primer is annealed to the template, then the mixture is split into four tubes and DNA polymerase and the four deoxynucleotide triphosphates (dNTPs), are added to each tube. Finally, a small amount of 2'3'-dideoxyadenosine triphosphate (ddATP) is added to the first tube, dideoxycytidine triphosphate (ddCTP) to the second, dideoxyguanosine triphosphate (ddGTP) to the third, and dideoxythimidine triphosphate (ddTTP) to the fourth. Polymerase extends the primer to form a chain complementary to the template. Occasionally the polymerase incorporates a dideoxynucleotide triphosphate (ddNTP) that causes termination of the chain extension since the ddNTP lacks a 3' hydroxyl group. The product of the chain extension and termination reaction in the first tube is a set of DNA fragments that have a common 5' end, defined by the primer, and that all terminate with dideoxyadenosine (ddA). The second reaction produces fragments ending in dideoxycytidine, the third, dideoxyguanosine, and the fourth, dideoxythymidine. This process is presented schematically in Figure 1.4. Sanger

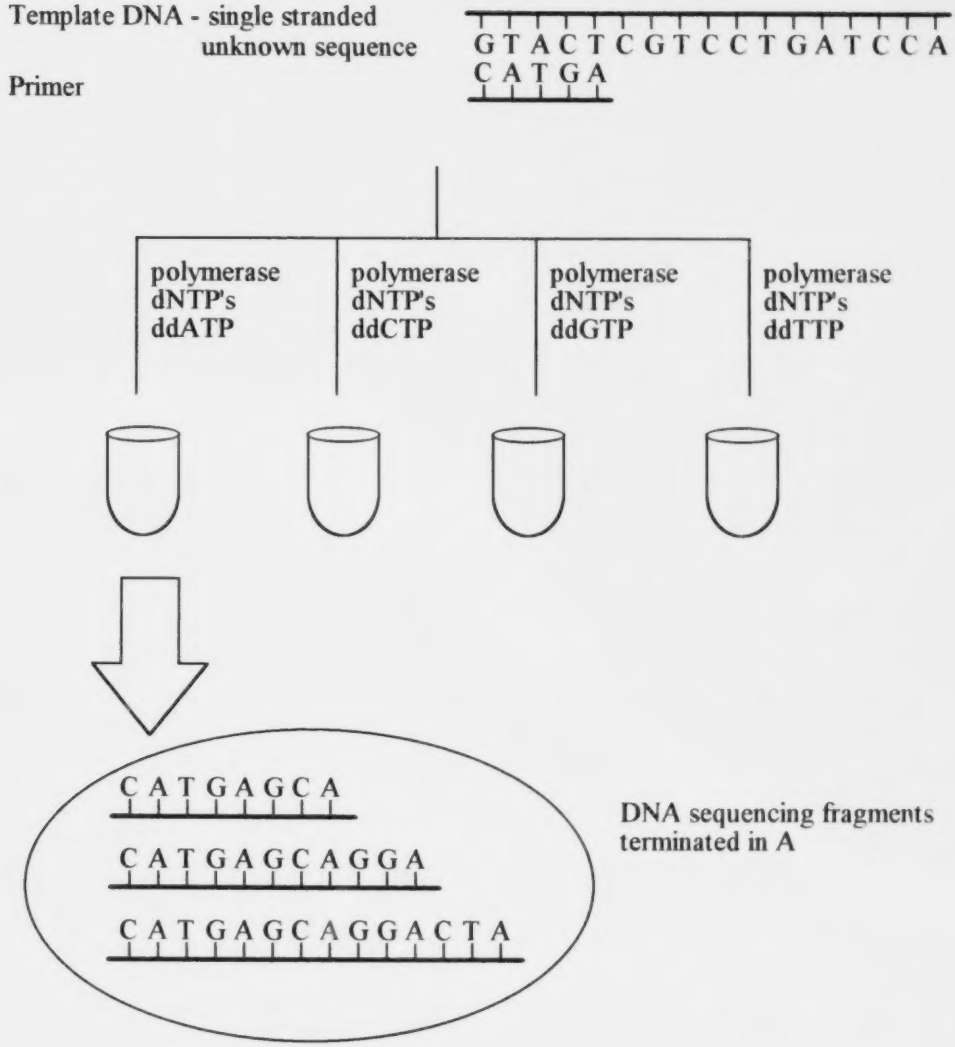


Figure 1.4 The chain extension and termination reactions - only the A sample is shown in detail.

labelled the DNA fragments by incorporation of a radioactively labelled deoxynucleotide in the synthesised DNA fragments. The DNA fragments are separated on the basis of size by polyacrylamide gel electrophoresis. The samples are loaded on four adjacent lanes of a rectangular slab of polyacrylamide. The application of an electric potential across the slab drives the separation. The negatively charged DNA fragments migrate down the gel. The smaller fragments migrate through the polymer network more quickly than the larger fragments. After a period of time the electrophoresis is stopped by turning off the electric potential. The bands of DNA are detected by exposing a film to the gel for a period of time, a process called autoradiography. The resulting autoradiogram gives the DNA sequence. The identity of the terminal nucleotide is indicated by the lane where the band is seen. The sequence is read from the bottom to the top of the autoradiogram (Figure 1.5).

1.2.2 The Maxam-Gilbert Chemical Degradation Technique

The Maxam-Gilbert technique for DNA sequencing relies on selective chemical degradation of the DNA to produce the nested set of fragments (5). The DNA is generally radiolabelled at one end to enable detection by autoradiography. It is then divided into five reaction tubes. The DNA in each tube is partially cleaved in a chemical reaction specific for one base e.g. G or type of base e.g. purine, A or G. The bases undergo chemical modification as summarised in Table 1.1, followed by cleavage of the sugar-phosphate backbone of the DNA by hot piperidine at the sites of chemical modification. The resulting cleavages occur for G, A+G, C+T, C and A>C. Partial

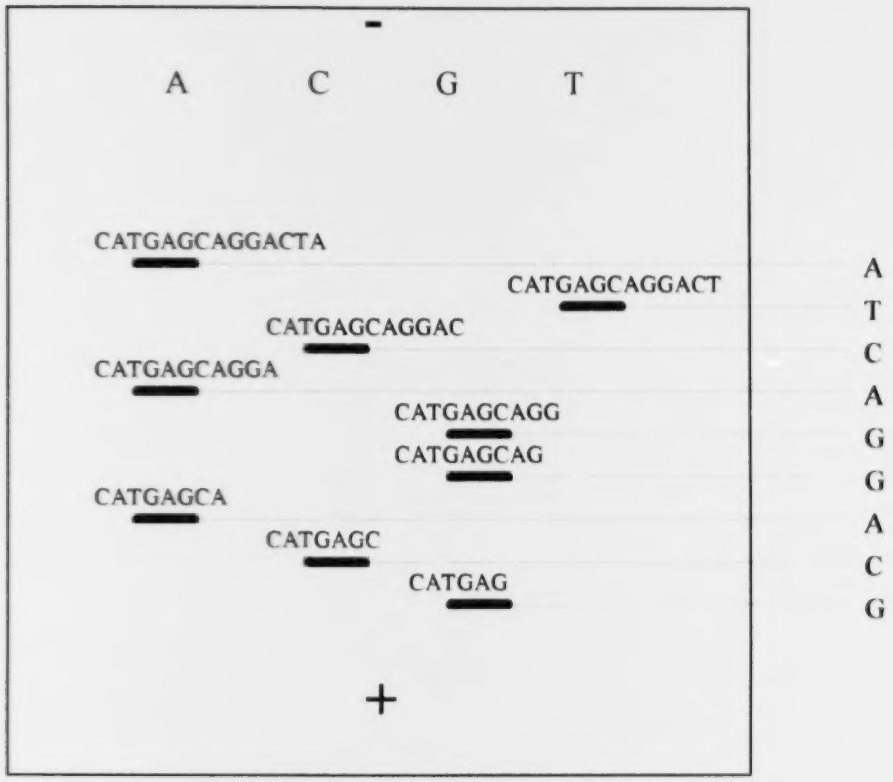


Figure 1.5 Slab gel electrophoresis. The four sequencing samples are loaded on adjacent lanes of the gel. The fragments migrate toward the positive electrode and are separated on the basis of size. After autoradiography, the film would appear similar to the diagram above, a series of dark bands. The corresponding DNA fragment is printed above each band for clarity. The identity of the 3' terminal nucleotide is determined from the lane where the band occurs. The sequence is read from bottom to top, since the shorter fragments migrate more quickly, hence move further toward the bottom of the gel.

cleavage yields a mixture of fragments, whose lengths are determined by the position of the bases(s) in the original DNA (Figure 1.6).

Table 1.1 Chemical Modifications for Maxam-Gilbert Sequencing (6)

Base	Reagent	Resulting Modification
G	dimethyl sulphate, pH 8.0	Makes C8-C9 bond susceptible to base cleavage
A+G	piperidine formate, pH 2.0	protonates N in purines resulting in depurination
C+T	hydrazine	opens pyrimidine rings, they recyclise, and are susceptible to removal
C	1.5M NaCl, hydrazine	only C will react as above
A>C	1.2M NaOH	strong cleavage at A, weaker cleavage at C

The products of the reactions are loaded on five adjacent lanes of a gel, separated by electrophoresis, and detected by autoradiography. Comparison of the lanes on the gel yields the DNA sequence (Figure 1.7). The Maxam-Gilbert method generally gives sequences up to 250 nucleotides from the radiolabelled end of the DNA (6).

Note that the chain termination method yields the complementary sequence of the of the starting DNA, while the Maxam-Gilbert method gives the sequence of the starting DNA directly.

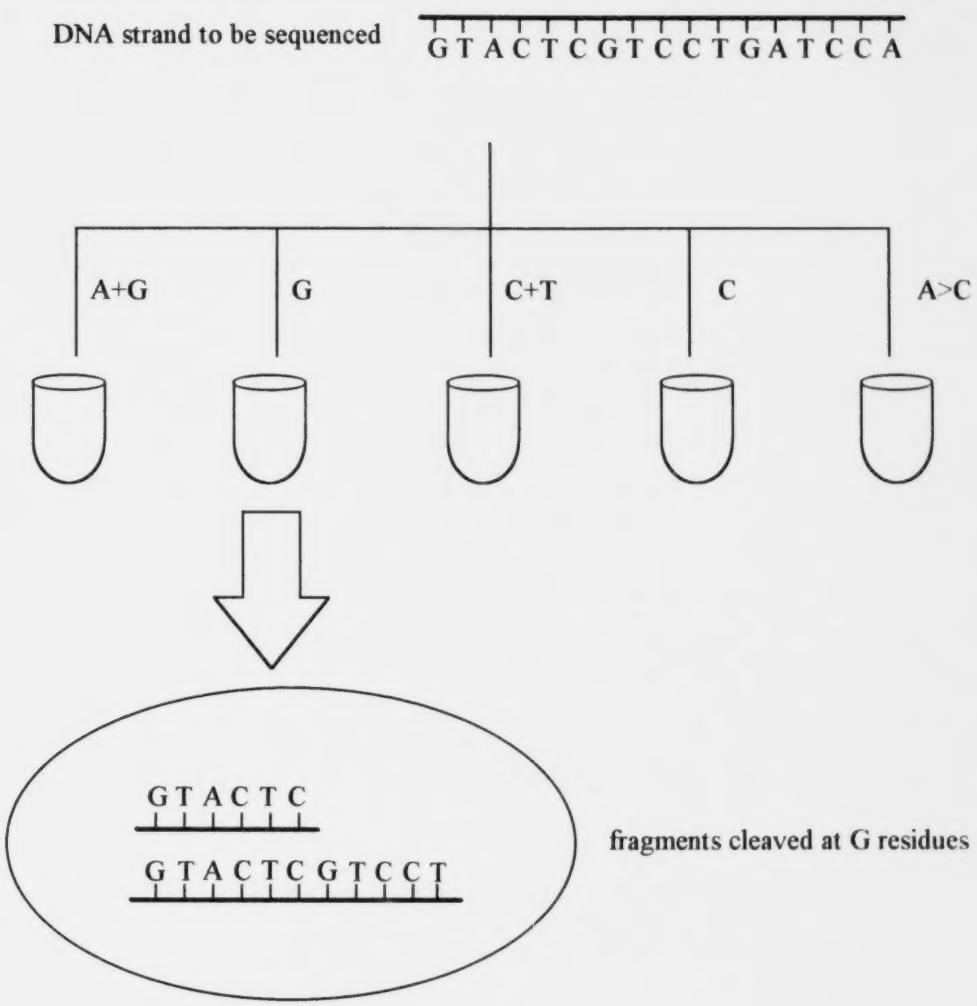


Figure 1.6 The Maxam-Gilbert chemical degradation reactions. The cleavage at the G residues are shown in detail.

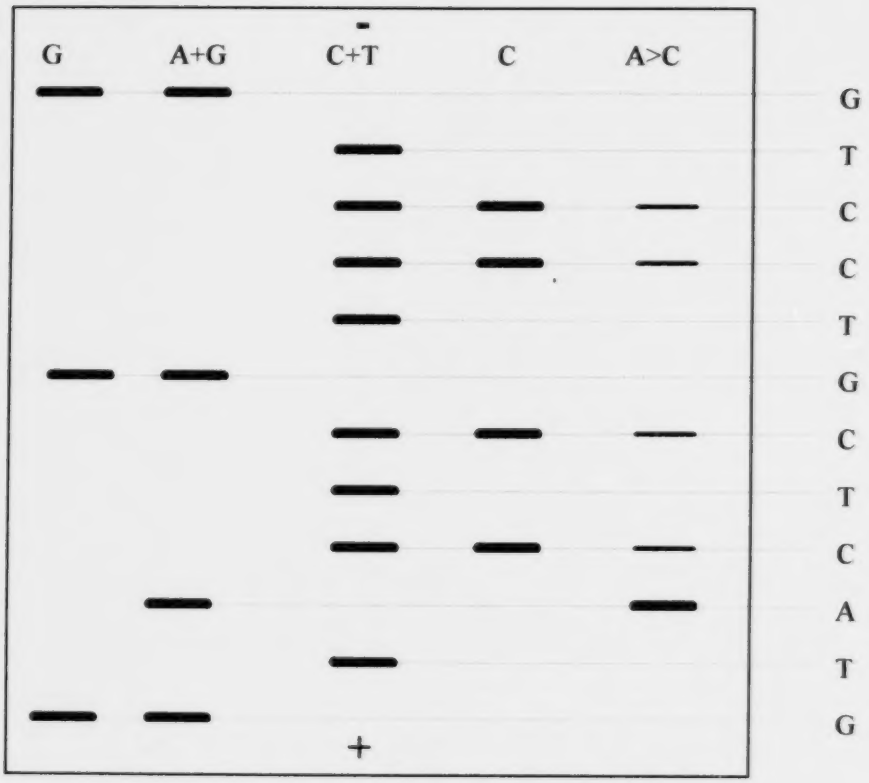


Figure 1.7 Slab gel electrophoresis of a Maxam-Gilbert sequencing sample. The DNA sequence is read from bottom to top. The identity of the base is established by comparison of the different lanes.

1.3 Modified Sequencing Approaches

While Sanger's method presented a major breakthrough in DNA sequencing, it is still labour intensive and relatively time consuming. Once the DNA samples have been prepared, approximately 8 to 16 hours is required for electrophoresis, 8 to 16 hours for autoradiography, and up to four hours to read and check the sequence. A typical sequencing run yields the sequence of 300 to 400 nucleotides. In addition to the time requirements, traditional sequencing methods require the use of radioactive nucleotides. In the mid 1980's the use of fluorescently labelled oligonucleotide primers was demonstrated by several different groups (7-9). This led to the development of automated DNA sequencers, which in a period of 8 to 12 hours perform electrophoresis, detect the fluorescent fragments, and determine the nucleotide sequence (10). The following sections provide an overview of the most widely implemented approaches to fluorescent labelling of DNA samples and the implications for instrument design.

1.3.1 One Fluorescent Primer

The simplest approach to fluorescent labelling is to incorporate a fluorescent label on the 5' end of the primer. This approach was demonstrated by Ansorge in 1986 (7). All samples have the same label, hence four separate sequencing reactions are carried out, and the products of the reactions are separated on four adjacent lanes of a slab gel. The identity of the terminal nucleotide is determined by the lane where the band is located, analogous to autoradiography. The hallmark of this method is its simplicity; only one label is used, simplifying detector optics since only one laser is required to excite the fluorescent label and only one detector channel is necessary.

The disadvantages are that four separate reactions are required, and four lanes on the gel are used for only one sample. Both Pharmacia and Hitachi have developed automated sequencers based on this approach.

1.3.2 Four Fluorescent Primers

The approach pioneered by Smith *et al.* (8). makes use of four spectrally distinct fluorescent labels. In this approach, the four individual sequencing reactions are carried out. Each reaction makes use of a primer with one of the four labels. The reaction products are then pooled and separated on a single lane of a slab gel. The detector optics for this system are more complicated. Two lasers are required to excite all four labels, and four detector channels are necessary. Four separate sequencing samples are still required, but only one lane on the gel is used. The use of four distinct labels minimizes ambiguities. The Applied Biosystems automated DNA sequencer is based on this approach.

1.3.3 Dye terminators

The use of fluorescent ddNTP's or dye terminators was first demonstrated by Prober *et al.* at DuPont (11). Each of the four ddNTPs is labeled with a fluorescent dye, and all four ddNTPs were added to one DNA sequencing reaction. The four dyes are derivatives of fluorescein with closely spaced excitation and emission spectra. All four dyes are excited by an argon ion laser operating at 488 nm. The fluorescence is split into two spectral channels and the identification of the terminal nucleotide was based on the ratio of the signals in the two channels. The advantages of this approach are first, that only one sequencing reaction is required as well as only one lane on the

gel, and second, that false terminations (those not caused by the incorporation of a ddNTP) are not detected. Only one laser, and two spectral channels are required for the detector. Unfortunately this method suffers from poor accuracy due to the close spacing of the fluorescence spectra. The instrument based on this approach was manufactured by DuPont, but is no longer available.

The same principle has been applied with four fluorescent dyes that have more widely spaced spectra, and which are compatible with the ABI sequencer (12). These dyes are excited by two different laser lines, and are detected in four different spectral channels. This approach maintains the advantage of one reaction and one lane on the gel combined with the more complicated detector design.

1.3.4 Internal Labelling with Fluorescent dNTPs

In 1992 Voss *et al.* first reported labelling of DNA samples with a fluorescein tagged dUTP (13). The fluorescein-dUTP was incorporated in a separate labelling step similar to that used to incorporate radioactive nucleotides. In a separate report, labelling with fluorescein-dATP was reported by the same group in 1993 (14). Samples produced in this manner are all tagged with the same label, hence four reactions and four lanes on the gel are required. The samples are suitable for separation on the Pharmacia sequencer. The main advantage of internal labelling is the avoidance of the costly and time consuming preparation of labelled primers. The labelled dNTPs are incorporated more efficiently than the labelled ddNTPs by the DNA polymerases used in sequencing.

1.3.5 Peak Height Methods

In 1990 Richardson and Tabor demonstrated a one label, peak height encoded method for DNA sequencing (15). The basis of this method is the very uniform pattern of terminations produced by T7 polymerase in the presence of manganese ions (16). Fluorescent primers are used to label the samples, and the reaction is carried out in one tube. The dideoxynucleotides are added in different amounts, for example with a ratio of 8:4:2:1 of ddATP:ddGTP:ddCTP:ddTTP. In this way, the proportion of fragments terminated by ddA is greatest and by ddC is the least. The DNA sequence is encoded in the intensity of the signal; the most intense signal corresponds to A, the least intense, T, and the intermediate levels, G and C (Figure 1.8). This approach requires only one sample preparation, one lane on the gel, and a simple detector design since only one label is used. These samples may also be internally labelled with fluorescent dNTPs (Sec. 1.3.4). The main drawback is that the accuracy is not as high as for other methods. Two types of errors occur: 1. ambiguities in the two bases coded by the intermediate peak heights, and 2. late in the run as the peak spacing decreases, and as the least intense peaks become very small they may be missed completely.

1.3.6 Cycle Sequencing

Cycle sequencing(17-18) is an offshoot of the polymerase chain reaction (PCR) (19). PCR results in an exponential amplification of a segment of DNA by repetitive cycling (Figure 1.9a). Each cycle consists of three steps: 1. the high temperature denaturation of double stranded DNA, 2. the annealing of two complementary primers that flank the segment of DNA to be amplified, and 3. the extension of the

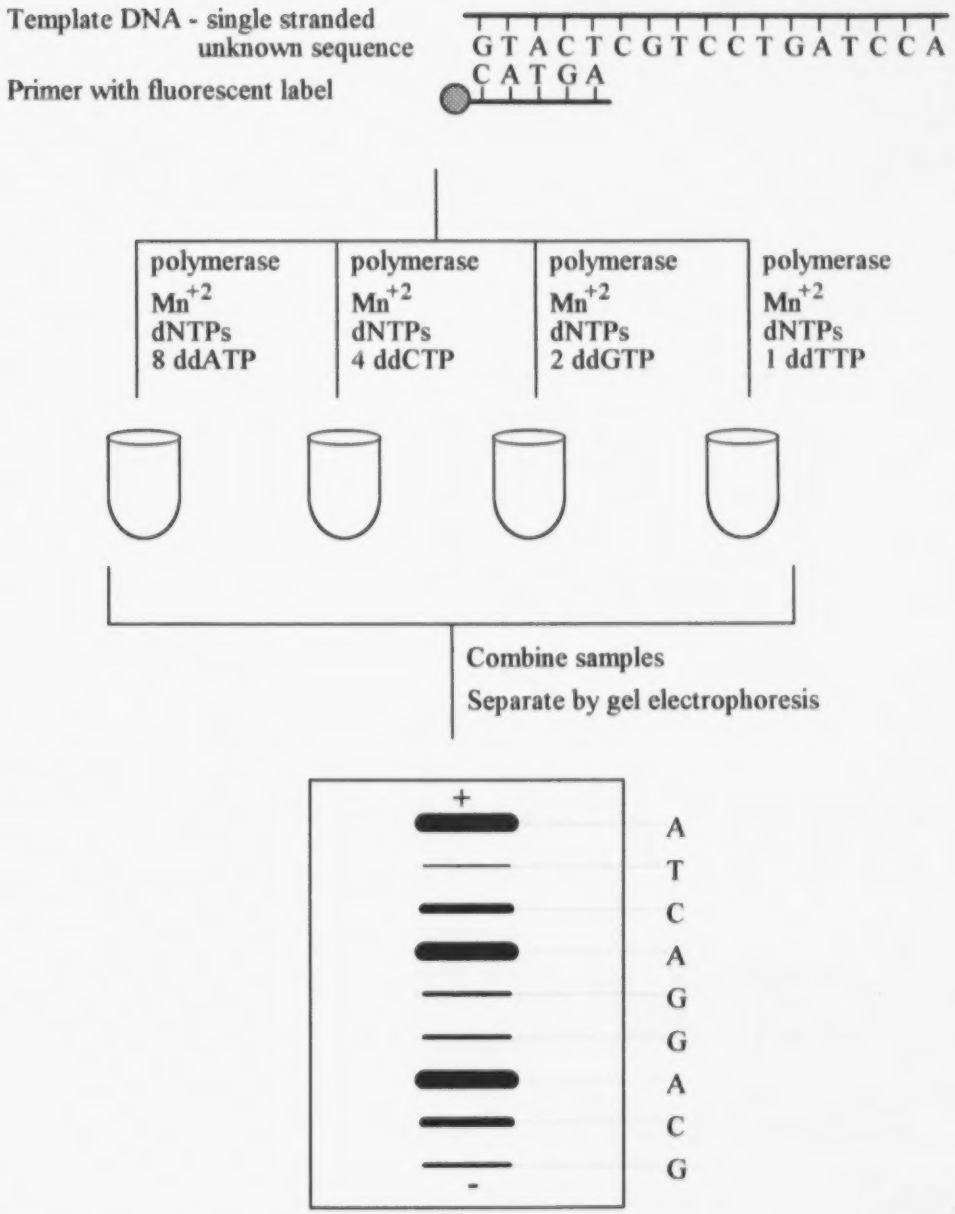


Figure 1.8 The peak height method developed by Richardson and Tabor. The ddNTP's are added in the ratio 8:4:2:1 A:C:G:T. The samples are combined and separated on a single gel lane. The intensity of each band identifies the 3' terminal nucleotide: The most intense bands are A's, the least intense are T's.

primers by DNA polymerase. The use of thermostable *Taq* polymerase, which withstands the denaturation step, was a significant advance (20). PCR allows very small amounts of DNA to be amplified to easily detectable amounts.

Cycle sequencing uses only one primer, to linearly amplify the target DNA, in the presence of ddNTPs that terminate each newly extended chain (Figure 1.9b). The amplified DNA is a nested set of fragments like those produced by Sanger's method (4). Amounts as low as nanograms of DNA template can be sequenced. Conventional sequencing reactions require microgram quantities of template DNA. In theory, cycle sequencing would avoid the time consuming preparation of large amounts of template.

1.4 Mapping and Sequencing the Genome

The human genome contains approximately 3×10^9 base pairs divided into 23 chromosomes. The genome also contains about 100 000 genes, the DNA sequences that code for proteins as well as information that regulates the kind and amount of protein made in a particular cell. The genes account for about 5% of the total DNA present in the genome, and they are interspersed with regions of non-coding DNA.

The goal of the Human Genome Project is to map and sequence the entire human genome within 15 years. A brief overview of some of the techniques used, and the steps necessary to get to DNA sequence starting from chromosomal DNA are presented below.

1.4.1 Restriction Digests

DNA molecules may be cut into fragments by restriction enzymes. Restriction enzymes are isolated from various types of bacteria. Each restriction enzyme cuts

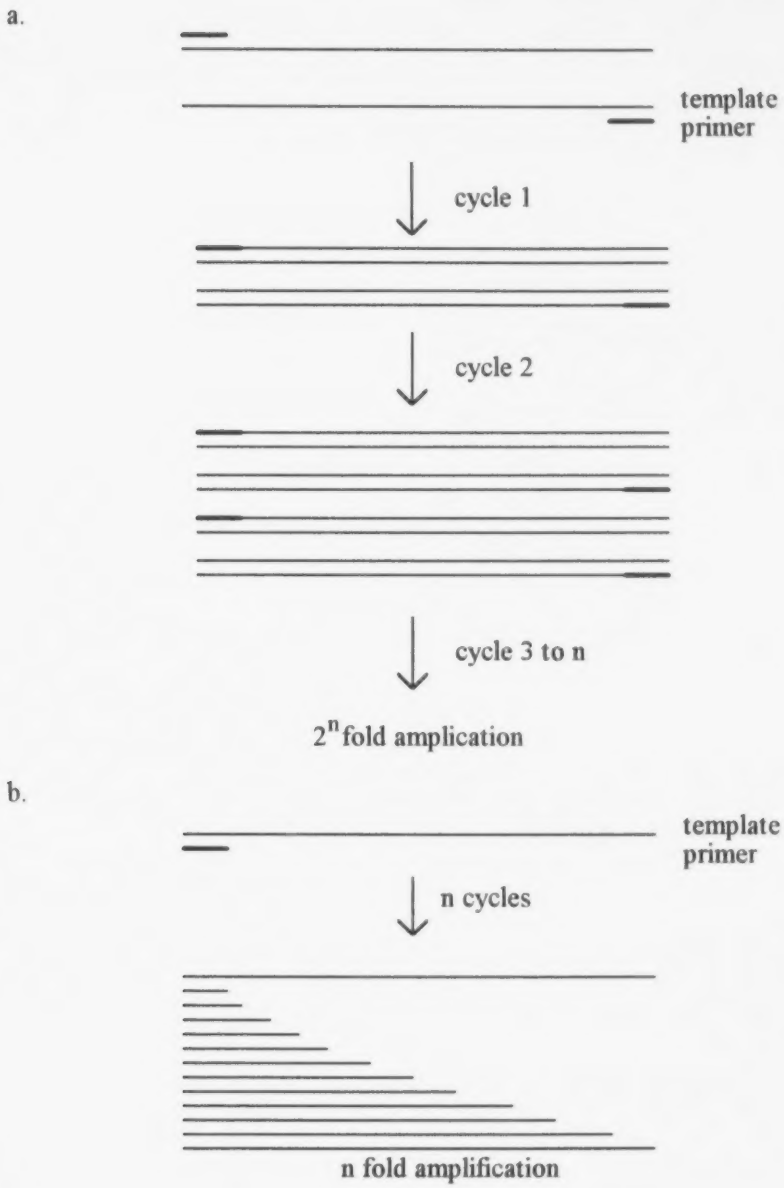


Figure 1.9 a. Polymerase chain reaction (PCR). Each successive cycle produces a twofold amplification of the target DNA.

b. Cycle sequencing. Each cycle produces a ddNTP terminated fragment resulting in a linear amplification and a nested set of fragments.

double stranded DNA at or adjacent to a unique recognition site. The enzyme recognition site may be 4 to 8 bases long. Some examples of restriction enzymes and their recognition sites are shown in Table 1.2 (21).

Table 1.2 Some restriction enzymes and their recognition sites. The cutting sites are indicated by the arrows.

Source	Enzyme	Recognition Sequence
Haemophilus aegyptius	*Hae*III	G↓CC CC↑GG
Escherichia coli RY13	*Eco*RI	G↓AATTC CTTAA↑G
Bacillus amyloliquefaciens H	*Bam*HI	G↓GATCC CCTAG↑G
Nocardia otitidis-caviarum	*Not*I	GC↓GGCCGC CGCCGG↑CG

If DNA is a random sequence of the four deoxynucleotides, then an enzyme with a 4 base recognition site will give, on average, fragments 256 nucleotides (or bases) in length, one with a 6 base recognition site will give 4000 base fragments, and one with an 8 base recognition site will give 64 000 base fragments. Hundreds of different restriction enzymes are known. The resulting restriction fragments are useful for mapping and cloning experiments.

1.4.2 Gel Electrophoresis

DNA fragments may be separated on the basis of size by gel electrophoresis. Fragments of up to 1000 nucleotides can be separated with polyacrylamide gels. Agarose gel will separate fragments ranging from 100 to 50 000 nucleotides, depending on the gel concentration (21). Longer fragments may be separated using

pulsed field gel electrophoresis. Individual bands may be recovered from the gel and purified for further experiments.

1.4.3. Cloning

DNA fragments may be inserted into a circular DNA molecule known as a cloning vector to produce a recombinant DNA molecule. The vector is introduced into a bacterial cell where it is multiplied. The copies of the vector which are produced all contain the inserted DNA fragment. When the bacterial host cell multiplies the recombinant DNA is passed on to the new cells. As cell division proceeds a large number of identical host cells results, each containing multiple copies of the recombinant DNA molecule. Suitable vectors include plasmids, bacteriophages (phages), cosmids, and yeast artificial chromosomes (YACs). Plasmids are small circles of DNA that are found in bacteria and can replicate independently of the bacterial chromosome. A bacteriophage is a virus that infects bacterial cells and is replicated inside the cells. Plasmid and phages hold DNA inserts of up to about 10 kilobases (kb). A cosmid is a hybrid between a phage and a plasmid that will hold inserts of up to 40 kb in size. YACs are composed of the structural components of the yeast chromosome. YACs can hold very large inserts. (22-23)

A series of clones is generally prepared from the DNA of interest, for example chromosomal DNA. The collection of overlapping clones that represents the entire length of the starting DNA is called a library.

1.4.4 Mapping and Sequencing

Mapping involves breaking the DNA down into fragments small enough to

characterise. These fragments are ordered, or mapped to correspond to their approximate positions on the chromosome. At the coarsest level is the chromosome map, where genes or fragments of DNA are assigned to one of the chromosomes. Generally this is accomplished by hybridisation techniques: a piece of tagged DNA (either fluorescent or radioactive) that is complementary to a region of the gene being mapped is used to find and bind to the complementary strand of DNA in the chromosome. Chromosome maps typically locate a DNA fragment within a region of about 10 million base pairs (24).

Genetic maps are constructed by observation of the pattern of inheritance of pairs of genetic markers. A useful genetic marker is any molecular or physical characteristic that is inherited and that differs between individuals. If two markers are located close to each other on the chromosome, they tend to be passed together from parent to child. Occasionally, DNA strands break and rejoin, an event known as recombination. The closer two markers are, the less likely they are to be separated by a recombination event. Currently, genetic maps have a resolution of about 10 million base pairs. The strength of a genetic map is that inherited diseases can be located on the map, aiding in finding disease genes.

Restriction maps are physical maps, ordered sets of DNA fragments, that are prepared by cutting the genomic DNA with restriction enzymes. The map shows the positions of different restriction sites on a DNA molecule. A chromosome may be cut into small pieces with restriction enzymes. Each of the pieces is cloned and the clones are arranged in serial fashion to give a contiguous map known as a contig. Alternatively, the chromosome may be cut with rare-cutter restriction enzymes to give large pieces, which are cloned in YACs or other vectors, and are ordered. The fragments are then subdivided and mapped further. This approach gives a more continuous map, but coarser resolution.

Once the map is constructed, each of the vectors is sub-cloned. Sub-cloning is carried out because YACs are too large to be sequenced directly. The inserts in the YACs are mapped, divided into smaller pieces, and cloned in a different vector, such as a cosmid. The cosmids are further subdivided and grown in plasmids. The plasmids, for example pBluescript, are the templates used for DNA sequencing and may be produced in the single stranded form. Generally, one of two sequencing strategies is employed. The first approach is random or shotgun sequencing. In this approach a large number of subclones are prepared containing random segments of target DNA (Figure 1.10a). Once the sequence data for the fragments is obtained, a computer orders the final sequence. In general, it is necessary to sequence 5 to 7 times the length of the target DNA to generate the entire sequence (6). The second approach is a directed approach, where the sequences of the target DNA are obtained in a systematic way. An example of a directed approach is primer walking which sequences stretches of DNA in a stepwise fashion (Figure 1.10b). The sequence from the first reaction is used to design the primer for the next reaction. The DNA sequence of the entire insert is obtained by moving the priming site along the DNA (6).

The raw sequence data is assembled to give a finished sequence which is then edited.

1.5 Capillary Electrophoresis

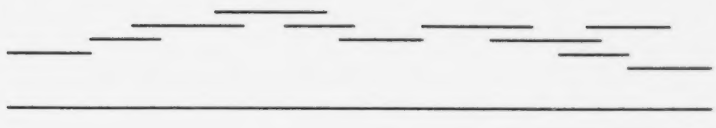
Electrophoresis is the separation of charged molecules based on their mobility in an applied electric field. Historically, electrophoresis has found widespread application in the separation of biological macromolecules such as proteins and nucleic acids. The efficiency of electrophoretic separations is limited by Joule heating which

causes convection. Usually electrophoresis is carried out with a solid support, *e.g.* paper, or with an anticonvective medium such as polyacrylamide gel. Hjerten first described open tubular zone electrophoresis (25). In 1983 Jorgenson and Lukacs described capillary electrophoresis (26), which has rapidly grown into a relatively mature field.

There are several advantages associated with capillary electrophoresis, which are related to the dimensions of the capillary. The small inner diameter of the capillaries, typically 10 to 100 μm, minimizes convection (27), removing the need for an anticonvective medium. The high surface-to-volume ratio dissipates heat efficiently, which allows application of high electric fields resulting in very fast and efficient separations. Separation efficiencies of up to 2.5 million theoretical plates are not uncommon in free zone capillary electrophoresis

The theory of open tubular capillary electrophoresis shows that the separation is based on the charge-to-radius ratios of the analytes. Application of free zone electrophoresis to separations of DNA is limited since the charge-to-radius ratio of DNA remains approximately constant as the chain size increases. A direct technology transfer was the key to separating DNA fragments by capillary electrophoresis. In 1987 Karger described the preparation of polyacrylamide gel filled capillary tubes (28). Swerdlow and Gesteland applied the gel filled capillaries to the separation of DNA sequencing samples (29). The gel acts as a sieving medium and separates the fragments on the basis of size. Traditionally, slab gel electrophoresis was carried out in crosslinked polyacrylamide gel. The composition of such gel is expressed as total

a.



b.

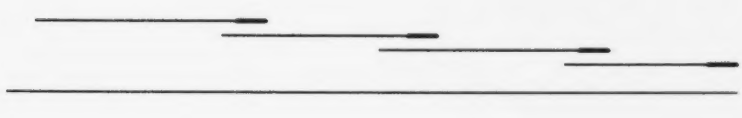


Figure 1.10 a. Random or shotgun sequencing. A series of random clones is generated and sequenced. The sequences of the fragments are ordered by a computer.

b. Primer walking. A portion of the target DNA is sequenced starting with a primer (shown by the short, heavy line). The newly obtained sequence is used to prepare a second primer, which is used to sequence the next portion of the target DNA. This is continued until the target DNA is sequenced.

percent acrylamide, %T, and percent crosslinker, %C.

$$\%T = \frac{W_{Acrylamide}(g) + W_{Bis}(g)}{V(ml)} \times 100\% \qquad (1\text{-}1)$$

$$\%C = \frac{W_{Bis}(g)}{W_{Acrylamide}(g) + W_{Bis}(g)} \times 100\% \qquad (1\text{-}2)$$

where the weights of acrylamide, $W_{Acrylamide}$, and the crosslinker N,N'-methylenebisacrylamide (Bis), W_{Bis}, are in grams, and the total volume of the solution is in millilitres. The mobility, μ, of a DNA fragment of a given length is sometimes described by a function of the gel concentration:

$$\log \mu = \log \mu_0 + k(\%T) \qquad (1\text{-}3)$$

where μ_0 is the mobility in free solution (%T=0), k is the retardation coefficient, and %T is the total acrylamide concentration, where crosslinker concentration, %C, is held constant. High resolution separations of oligonucleotides are also carried out in non-crosslinked polyacrylamide (30).

1.6 Laser-Induced Fluorescence

The impressive speed and efficiency of separations performed by capillary electrophoresis are directly linked to the small dimensions of the capillary. Ironically, the capillary's dimensions and shape also produce the biggest challenge in implementing capillary electrophoresis, that of detection. A capillary, typically with dimensions of 50 μm i.d. and 50 cm long, has a volume of less than one microlitre. The concentration of analyte must be appreciably lower than the ionic strength of the

separation buffer in order to produce efficient, reproducible separations. As a result the volume of sample injected into the capillary is on the order of one nanolitre or less. Very high sensitivity detectors are required and include laser induced fluorescence (31), laser induced thermo-optical absorbance (32), electrochemistry (33), UV absorbance (34), radioactive isotopes (35), and mass spectrometry (36). The sensitivity of laser-induced fluorescence is sufficient to allow detection of very small amounts of samples.

The fluorescence intensity, I, is expressed as

$$I = QI_0(1 - 10^{-abc}) \qquad (1\text{-}4)$$

where Q is the quantum yield of the fluorophore, I_0 is the incident power of the excitation source, a is the molar absorptivity of the molecule, b is the sample path length, and c is the sample concentration. If the sample concentration is sufficiently low the following approximation is true

$$I = QI_0abc \qquad (1\text{-}5)$$

Equation 1-5 is further modified to

$$I = KQI_0abc \qquad (1\text{-}6)$$

Equation 1-6 includes an instrumental factor K, where $K<1$, reflecting the fact that fluorescence is emitted in all directions but is only viewed within a limited aperture. Capillary separations require that the sample concentration, c, and the path length, b, are small. The sensitivity may be increased by choice of a molecule with high quantum

yield Q and high molar absorptivity a, by matching the excitation source to the absorption maximum, increasing the source intensity I_0, and increasing the collection efficiency, which is related to K.

Laser induced fluorescence is ideal for detection in capillary electrophoresis. The spatial coherence of the laser beam allows it to be very tightly focussed to illuminate very small sample volumes with high intensity, I_0. When coupled with efficient collection and detection, very small volume samples may be detected.

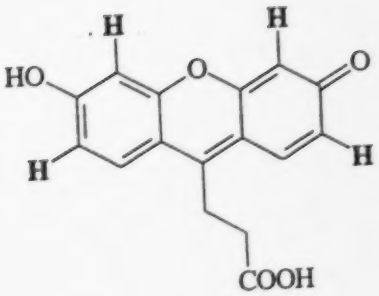
There are several sources of background signal in fluorescence measurements: fluorescence from impurities in the solvent, fluorescence from cuvette windows, Raman and Rayleigh scatter from the solvent, light scatter at the cuvette-sample interface (37) and detector dark current. Careful choice of reagents and sample preparation minimize background from the first source. Solvent Raman scatter is rejected by appropriate spectral filtering. Fluorescence and light scatter from the cuvette windows are minimized by spatial filtering, which restricts the field of view of the detector to the illuminated sample stream. If fluorescence detection in capillaries is performed on-column, light scatter from the capillary window is the major source of background signal. Use of the sheath flow cuvette as a post-column detector eliminates light scatter from the capillary interface, and lowers the background signal to nearly Raman scatter or even dark current limited values (38-39).

Most samples are not fluorescent and must be tagged with an appropriate label in order to be detected. Some popular labels are fluorescein and tetramethylrhodamine. Fluorescein has an absorbance maximum at 494 nm and is efficiently excited by the argon ion laser line at 488 nm. Tetramethylrhodamine, with an absorbance maximum at 547 nm, is excited by the green helium-neon laser line at 543.5 nm. A variety of samples have been labelled using these compounds, separated by capillary electrophoresis and detected by laser-induced fluorescence. Samples

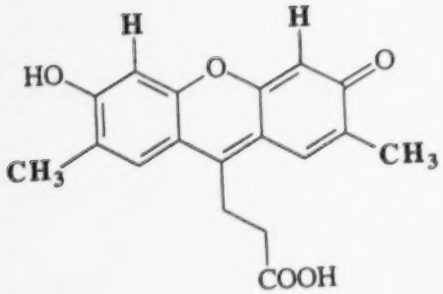
include amino acids (31), peptides (40), monosaccharides (41), and oligonucleotides (39).

A variety of labels have been used for the detection of DNA sequencing samples. The one-colour approach (sec 1.3.1) generally uses fluorescein (Figure 1.12). The dye terminator approach developed by DuPont (sec 1.3.3) used several modified fluorescein compounds (Figure 1.11). The dye terminator approach developed by ABI uses four different dyes with more widely spaced spectra. The four colour labelled primer approach (sec. 1.3.2) also uses four distinct dyes (Figure 1.12). The work described in this thesis uses a variety of labels. The DuPont dye terminators are used in Chapter 2, two of the ABI dye primers (FAM and JOE) in Chapters 3 and 4, and fluorescein and tetramethylrhodamine, in Chapter 5.

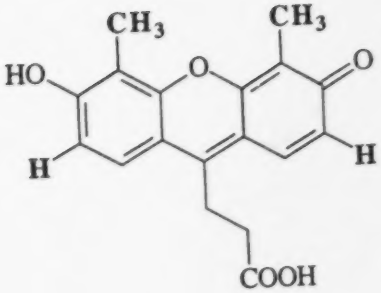
This thesis describes the effect of acrylamide concentration, %T, on the separation of DNA fragments by capillary electrophoresis, and describes a variety of approaches to DNA sequencing by capillary electrophoresis with laser induced fluorescence detection.



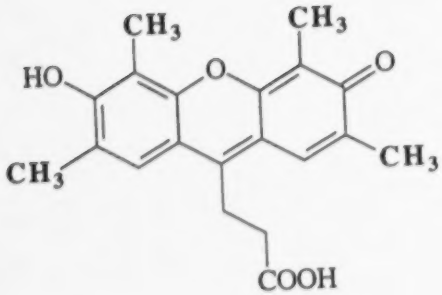
SF-505



SF-512

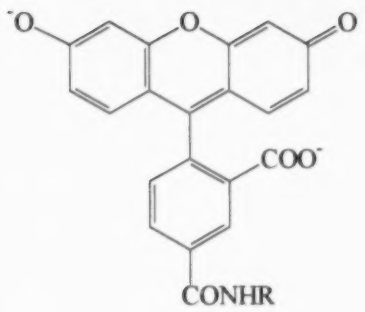


SF-519



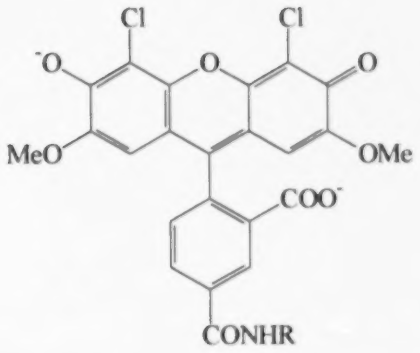
SF-526

Figure 1.11 The succinylfluorescein dyes used in the DuPont dye terminators. The number under each structure indicates the position (nm) of the emission maximum.



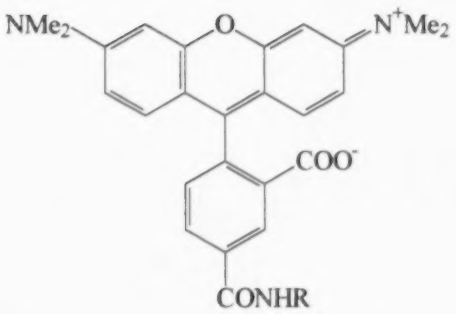
FAM (fluorescein)

Abs max = 494nm
Em max = 520nm



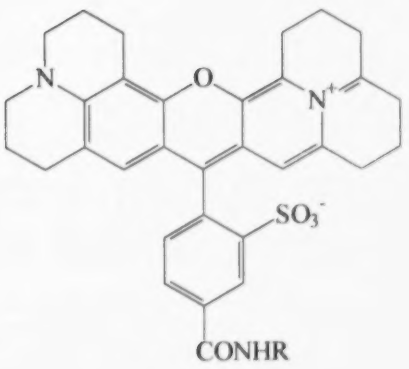
JOE

Abs max = 524nm
Em max = 550nm



TAMRA (tetramethylrhodamine)

abs max = 547nm
em max = 572nm



ROX

abs max = 589nm
em max = 615 nm

Figure 1.12 Fluorescent dyes used in the ABI dye primers.

References

1. H.F. Judson, *The Eighth Day of Creation*, New York, Simon & Schuster, 1979.
2. O.T. Avery, C.M. MacLeod, M. McCarty, *J. Exptl. Med.*, **79** (1944), 137.
3. J.D. Watson, F.H.C. Crick, *Nature*, **171** (1953), 737.
4. F. Sanger, S. Nicklen, A.R. Coulson, *Proc. Natl. Acad. Sci. USA*, **74** (1977), 5463.
5. A.M. Maxam, W. Gilbert, *Proc. Natl. Acad. Sci.*, **74** (1977), 560.
6. J. Sambrook, E.F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Ed., Plainview, NY, Cold Spring Harbor Laboratory Press, 1989.
7. W. Ansorge, B.S. Sproat, J. Stegemann, C. Schwager, *J. Biochem. Biophys. Meth.*, **13** (1986), 315
8. L.M. Smith, J.Z. Sanders, R.J. Kaiser, P. Hughes, C. Dodd, C.R. Connell, S.B.H. Kent, L.M. Hood, *Nature*, **321** (1986), 674.
9. H. Kambara, T. Nishikawa, T. Katayama, T. Yamaguchi, *BioTechnology*, **6** (1988), 816.
10. T. Hunkapillar, R.J. Kaiser, B.F. Koop, L. Hood, *Science*, **254** (1991), 59.
11. J.M. Prober, G.L. Trainor, R.J. Dam, F.W., Hobbs, C.W. Robertson, R.J. Zagursky, A.J. Cocuzza, M.A. Jensen, K.R. Bauermeister, *Science*, **238** (1987), 336.
12. L.G. Lee, C.R. Connell, S.L. Woo, R.D. Cheng, B.F. McArdle, C.W. Fuller, N.D. Halloran, R.K. Wilson, *Nucl. Acids Res.*, **20** (1992), 2471.
13. H. Voss, C. Schwager, U. Wirkner, J. Zimmermann, H. Erfle, N.A. Hewitt, T. Rupp, J. Stegemann, W. Ansorge, *Meth. Molec. Cell. Biol.*, **3** (1992), 30.

14. H. Voss, S. Wiemann, U. Wirkner, C. Schwager, J. Zimmermann, J. Stegemann, H. Erfle, N.A. Hewitt, T. Rupp, W. Ansorge, *Meth. Molec. Cell. Biol.*, **3** (1992), 153.

15. S. Tabor, C.C. Richardson, *J. Biol. Chem.*, **265** (1990), 8322.

16. S. Tabor, C.C. Richardson, *Proc. Natl. Acad. Sci. USA*, **86** (1989), 4076.

17. A.M. Carother, G. Urlaub, J. Mucha, D. Grunberger L.A. Chasin, *BioTehniques*, **7** (1989), 494.

18. V. Murray, *Nucl. Acids Res.*, **17** (1989), 8889.

19. K. Mullis, F.A. Faloona, *Methods Enzymol.*, **155** (1987, 335.

20. R.K. Saiki, D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, *Science*, **239** (1988), 487.

21. D.A. Micklos, G.A. Freyer, *DNA Science: A First Course in Recombinant DNA Technology*, 1990, Cold Spring Harbor Laboratory Press, .

22. T.A. Brown, *Gene Cloning: An Introduction*, Second Ed., London, Chapman & Hall, 1990.

23. T.A. Brown, *Genetics: A Molecular Approach*, Second Ed., London, Chapman & Hall, 1992.

24. C. Cantor, S.Spengler, *Primer on Molecular Genetics*, published in DOE *Human Genome 1989-90 Program Report*, revised and expanded by D. Casey, modified for Web access by D. Jacobson. Mosaic: http:\\wwwgdb.org\Dan\DOE\intro.html

25. S. Hjerten, *Chromatogr. Rev.*, **9** (1967), 122.

26. J.W. Jorgenson, K.D. Lukacs, *Science*, **222** (1983), 266.

27. F.E.P. Mikkers, F.M. Everaerts, J.P.E.M. Verheggen, *J. Chromatogr.*, **169**, 11-20 (1979).

28. A.S. Cohen, B.L. Karger, *J. Chromatogr.*, **397** (1987), 409.

29. H. Swerdlow, R. Gesteland, *Nucl. Acids Res.*, **18** (1990), 1415.

30. M.C. Ruiz-Martinez, J. Berka, A. Belenkii, F. Foret, A.W. Miller, B.L. Karger, *Anal. Chem.*, **65** (1993), 2851.

31. Y.F. Cheng, N.J. Dovichi, *Science*, **242** (1988), 562.

32. M.Yu, N.J. Dovichi, *Appl. Spectrosc.*, **43** (1989), 196.

33. R.A. Wallingford, A.G. Ewing, *Anal. Chem.*, **60** (1988), 1973.

34. Y. Walbroehl, J.W. Jorgenson, *J. Chromatogr.*, **315** (1984), 135.

35. S.L. Pentoney, R. Zare, J. Quint, J. Chromatogr., (1989), 480, 259-270.

36. R.D. Smith, J.A. Olivares, N.T. Nguyen, H.R. Usdeth, *Anal. Chem.*, **60** (1988), 436.

37. C.A. Parker, *Photoluminescence of Solutions*, New York, Elsevier, 1968, pp. 411-426.

38. S. Wu, N.J. Dovichi, *J. Chromatogr.*, **480** (1989), 141.

39. D.Y. Chen, H.P. Swerdlow, H.R. Harke, J.Z. Zhang, N.J. Dovichi, *J. Chromatogr.*, **559** (1989), 237.

40. J.Y. Zhao, K.C. Waldron, J. Miller, J.Z. Zhang, H. Harke, N.J. Dovichi, *J. Chromatogr.*, **608** (1992), 239.

41. J.Y. Zhao, N.J. Dovichi, O. Hindsgaul, S. Gosselin, M.M. Palcic, *Glycobiology*, **4** (1994), 239-242.

CHAPTER 2

The Effect of Total Percent Polyacrylamide in Capillary Gel Electrophoresis for DNA Sequencing of Short Fragments[1]

2.1 Introduction

DNA sequencing is based on the separation of labelled DNA fragments by denaturing gel electrophoresis. The rate of separation of the fragments is proportional to the electric field strength; high electric fields produce fast separations. In practice the applied field strength is limited by Joule heating which causes temperature gradients in the gel. Because the mobility of DNA fragments depends strongly on temperature, 2.3% per degree, thermal gradients lead to band broadening which degrades the separation efficiency in slab gels at high electric field strengths (1).

Conventional slab gels are about 0.5 mm thick. Thin slab gels, about 0.1 mm thick, generate fast and efficient separations at high fields (2-4). However, difficulties in automation, in maintaining a uniform gel thickness across the slab, and in detection have retarded widespread applications of this technology. On the other hand, capillary gel electrophoresis offers highly uniform chambers and high sensitivity detection technology. Typical fused-silica capillaries of 50 μm inner diameter produce outstanding thermal properties. Finally, the highly flexible nature of the capillaries simplifies automation.

Several groups have developed DNA sequencers based on capillary gel electrophoresis and laser-induced fluorescence detection (4-11). In these systems, the capillaries are filled with denaturing polyacrylamide gels. The sequencing rate observed in these gels depends on the details of the gel composition. This chapter

---

[1] A version of this chapter has been published in *Journal of Chromatography*, **608** (1992),143-150.

describes and models the sequencing rate, resolution, and separation efficiency in DNA sequencing by capillary gel electrophoresis.

2.2 Experimental

2.2.1 Instrument Design

A schematic of the instrument is shown in Figure 2.1a. Polyimide coated fused silica capillaries (Polymicro, Phoenix, AZ, USA) were typically 35 cm long, 190 μm outer diameter (o.d.) and 50 μm inner diameter (i.d.). The capillaries were filled with gel as described below. A Plexiglas box equipped with a safety interlock holds the injection end of the capillary and the high voltage electrode. Negative high voltage is applied to the capillary to drive the DNA fragments through the capillary. The other end of the capillary is inserted into a quartz sheath flow cuvette (Precision Cells, NY, USA) (figure 2.1b). The sheath flow cuvette has 2 mm thick quartz walls and a 200 μm square flow chamber. The cuvette sits in a locally constructed holder which is held at ground potential. The sheath flow buffer, supplied by a high pressure syringe pump (Isco), flows into the cuvette around the capillary, and out the bottom. Fluorescence is excited with a 10 mW argon ion laser beam (Uniphase, CA, USA) operating at 488 nm, which passes just below the tip of the capillary (Figure 2.1b). As the DNA fragments exit the capillary they are entrained in the flowing sheath stream and pass through the laser beam. The fluorescence is collected at 90 degrees to the laser beam with a microscope objective (32X, 0.6 N.A., Leitz/Wild, Calgary, AB, Canada), imaged onto a 0.75 mm pinhole , passed through a 525 nm bandpass filter (Omega, VT, USA) to a 525 nm dichroic filter. Long wavelengths are transmitted, short wavelengths reflected. In both spectral channels the signals are collected with photomultiplier tubes (Hamamatsu, CA, USA). The current from each photomultiplier tube is dropped

a

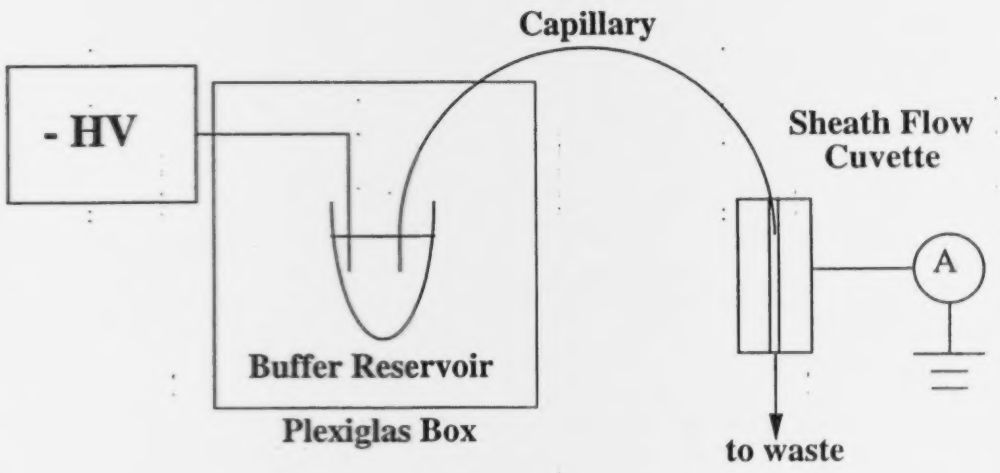


Figure 2.1a Schematic diagram of the capillary electrophoresis instrument.

b

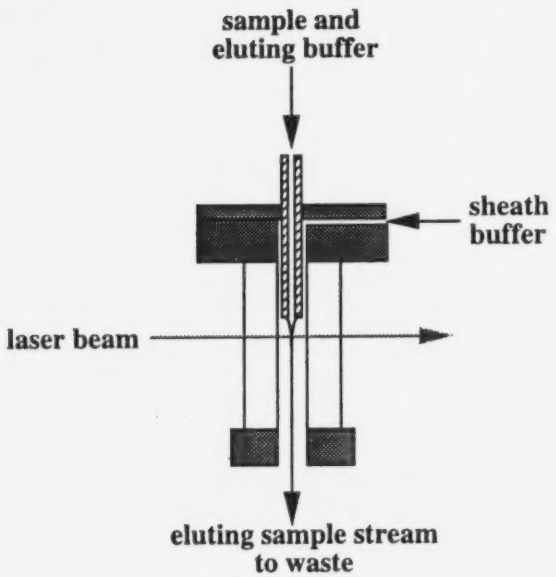


Figure 2.1b The sheath flow cuvette.

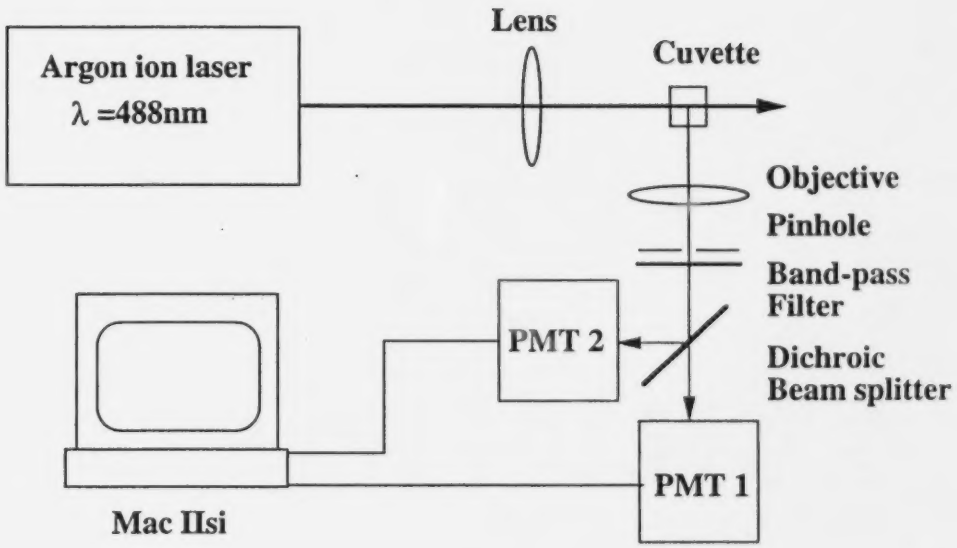


Figure 2.2 Schematic diagram of the one-laser two-channel fluorescence detector.

across a resistor, digitised, and recorded with a Macintosh IIsi computer. For this study, only the signal in the transmitted channel was used.

2.2.2 Gel Preparation

Gels were prepared in 5 ml aliquots from mixtures of acrylamide and N,N'-methylenebisacrylamide (Bis) (Bio-Rad, Toronto, ON, Canada), 1X TBE (89 mM Tris (ICN, Montreal, PQ, Canada), 89 mM borate (ICN) and 2 mM EDTA (Sigma, St. Louis, MO)) and 7 M urea (ICN). The percentage of total acrylamide in the gel, %T, was varied from 2.5 to 6%, while the percentage of acrylamide present as crosslinker (Bis), %C, was held constant at 5%. The monomer solution was carefully degassed by application of vacuum for 20 min. while the solution was stirred. Polymerisation was initiated by the addition of 2 μl of N,N,N',N'-tetramethylethylenediamine (TEMED) and 20 μl of 10% ammonium persulphate. The gel solution was injected into the capillary with a modified syringe. The gel was covalently bound to the last *ca.* 5 cm of the capillary with a solution of 5% (v/v) γ-methacryloxypropyltrimethoxysilane in 50% acetic acid. The end of the capillary was dipped in the silanising solution for approximately 30 sec. so that the solution filled about 5 cm of the capillary. The capillary was then filled with the gel solution. Binding the gel to the capillary walls prevented it from extruding out of the capillary into the detection cuvette. Even though polymerisation appeared to be complete within 30 minutes, gels were typically stored overnight before use. The capillaries were inspected under a microscope to ensure that they were free of bubbles before installing them in the capillary electrophoresis apparatus.

2.2.3 Sample Preparation

The DNA sequencing reaction was carried out in 40 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, and 50 mM NaCl. Three microlitres of M13mp18 single stranded template DNA (1 mg/ml) was mixed with 25 ng of -21 M13 universal primer (United States Biochemical Corp., Cleveland, OH, USA). Deoxynucleotide triphosphates (dNTPs) and fluorescently labelled dideoxyadenosine triphosphate (ddATP) (DuPont, DE, USA) were added in a 10:1 ratio of dNTP:ddATP. Ten units of Sequenase 2.0 (USB) were added. The sample was incubated at 37°C for 10 min to carry out the chain extension and termination reactions, then at 90°C for 10 minutes to inactivate the Sequenase. The sample was then passed through a Sephadex G-50 spin column (Sigma, St. Louis, MO) to remove the unincorporated labelled ddATP, precipitated with 98% ethanol, and redissolved in 4.0 µl of a mixture of formamide-0.5 M EDTA (49:1), pH 8.0.

2.2.4 Electrophoresis

Samples were injected for 30 seconds at 150 V/cm. The sample was replaced with a fresh vial of 1X TBE and electrophoresis continued at 150 V/cm. The sheath flow stream was 1X TBE at a flow rate of 0.10 ml/h. Time was measured from the application of the separation potential. Two to three replicate runs were done on each capillary.

2.3. Results and Discussion

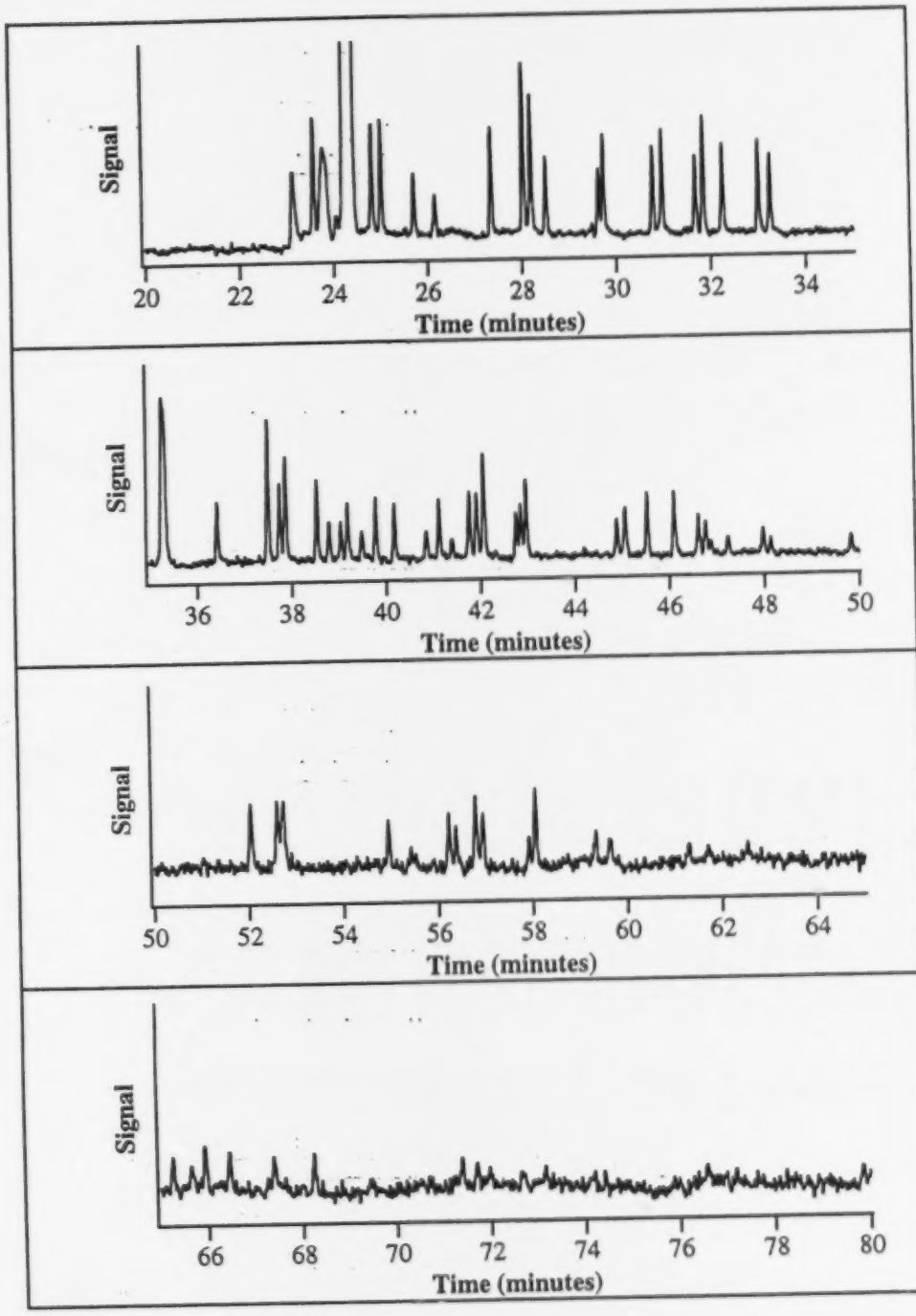
2.3.1 Performance and Stability of the Gel Filled Capillaries

Of the gel filled capillaries prepared in the manner described above, about 95% were useable. The 5% that were spoiled generally contained bubbles in the interior of the capillary. Bubbles in the gel interfered with electrophoresis since no current would flow through the capillary.

To prevent the gel from migrating out of the capillary into the detection cuvette under the influence of the high voltage, the inner wall of the capillary was treated with a silanising agent. The silanising agent, γ-methacryloxypropyltrimethoxysilane covalently bound the gel to the capillary walls. Treatment of the whole length of the capillary with the silanising agent was effective only if the total capillary length was less than 30 cm. When silanised capillaries longer than 30 cm were filled with acrylamide solution, small bubbles formed at regular intervals as the polyacrylamide gel shrank during polymerisation. Shrinkage of the gel at the ends of the shorter capillaries was presumably adequate to avoid the formation of bubbles. Silanisation of the last 2 to 5 cm of the detection end of the capillary prevented migration of the gel while allowing the preparation of capillaries longer than 30 cm.

On average, three sample injections were made on each gel filled capillary used in this study. The injection end of each capillary was trimmed between sample injections to minimize formation of bubbles at the end of the capillary . The gels were generally stable for the duration of three runs.

Figure 2.3 Electropherogram of an A-terminated M13mp18 sequencing sample.


Signal
20
22
24
26
28
30
32
34
Time (minutes)
Signal
36
38
40
42
44
46
48
50
Time (minutes)
Signal
50
52
54
56
58
60
62
64
Time (minutes)
Signal
66
68
70
72
74
76
78
80
Time (minutes)

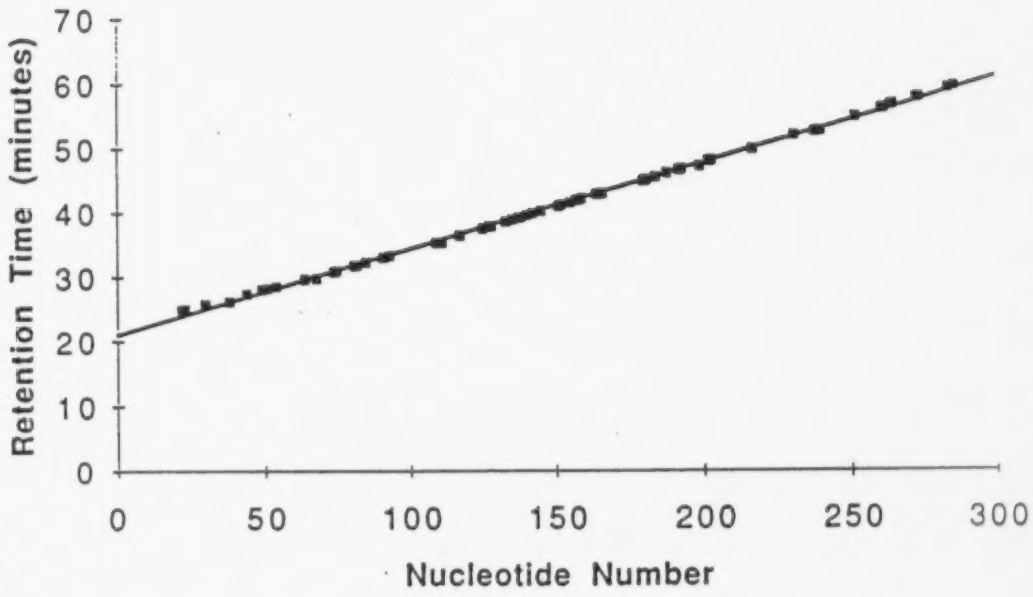


Figure 2.4 Retention time as a function of fragment length for single stranded DNA sequencing fragments separated in a 4%T, 5%C gel at an electric field strength of 150 V/cm in a 50 μm i.d., 35 cm long fused silica capillary. The separation was carried out at room temperature.

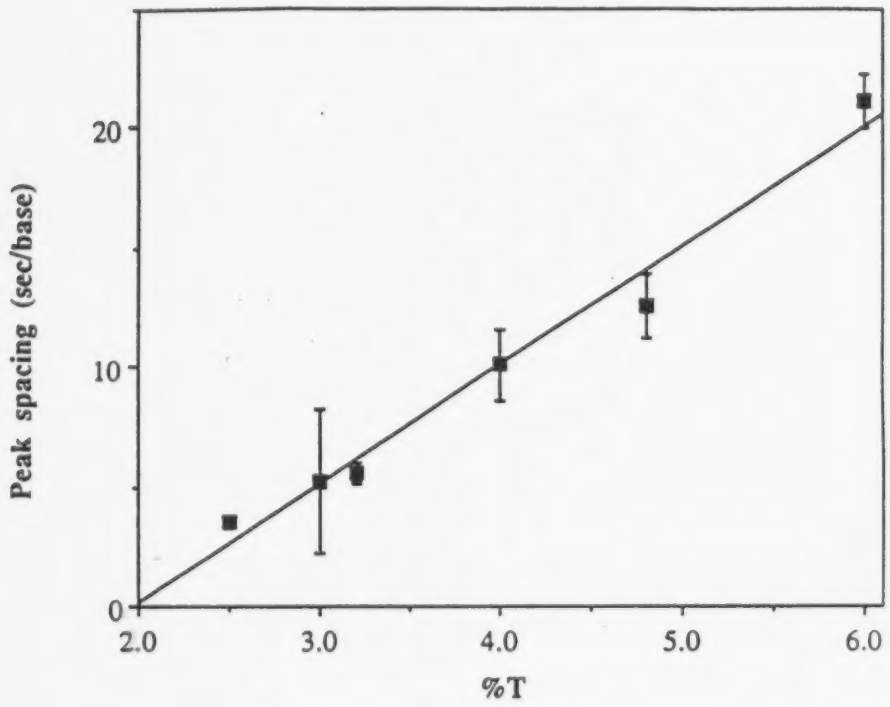


Figure 2.5 Peak spacing of DNA fragments for denaturing polyacrylamide gels with constant 5%C, room temperature operation, an electric field strength of 150 V/cm, and a 35 cm long 50 μm i.d. capillary. The data are shown at the 95% confidence interval and the line is the unweighted least squares fit with a linear function.

2.3.2 Sequencing Rate and Retention Time

Analysis was limited to 250 bases by the signal-to-noise ratio produced by this DNA sample. Figure 2.3 shows a typical electropherogram. The pattern of the peaks corresponds to the distribution of A's in the DNA sequence. In all cases, a plot of retention time *vs.* fragment length (in bases) was linear ($r>0.998$) for fragments ranging in size from the primer to at least 250 bases; that is,

$$\text{retention time} = t_0 + M \cdot \text{spacing} \qquad (2\text{-}1)$$

where t_0 is the intercept, spacing is the peak spacing in s/base, and M is the fragment length in bases. Figure 2.4 presents typical data for a 4%T gel; for these data, t_0 is 21.0 min. and the peak spacing is 8.0 s/base.

The peak spacing increased linearly with %T for all fragments (Figure 2.5). The data are shown at the 95% confidence interval; the line is the result of an unweighted least-squares fit. Only two runs were made with the 3% gel, resulting in a large confidence interval. The slope of this line, 5.0 s/base per %T, implies that the peak spacing increases by 5 s for every 1% increase in total acrylamide concentration. The intercept, -9.9 s/base, should be related to the free solution mobility of the DNA fragments. The negative sign implies that longer fragments will have a higher mobility than shorter fragments. The data of Kambara *et al.* (12) show a quadratic dependence of peak spacing on %T from 2 to 12%T. From 2 to 6%T, their data are similar to ours.

The inverse of peak spacing is sequencing rate (Figure 2.6). The data in bases/h are shown at the 95% confidence interval. The sequencing rate observed for the 2.5% gel was 1040 ± 10 bases/h, which is equal to the highest speed DNA

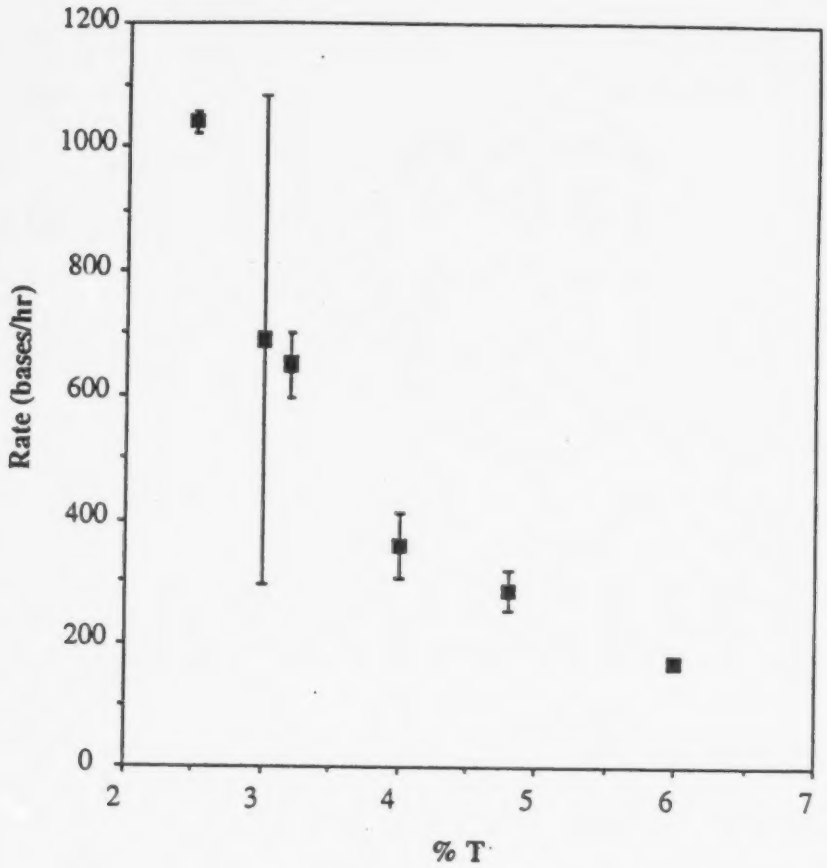


Figure 2.6 Sequencing rate for the data of Figure 2.4. Data shown at the 95% confidence interval.

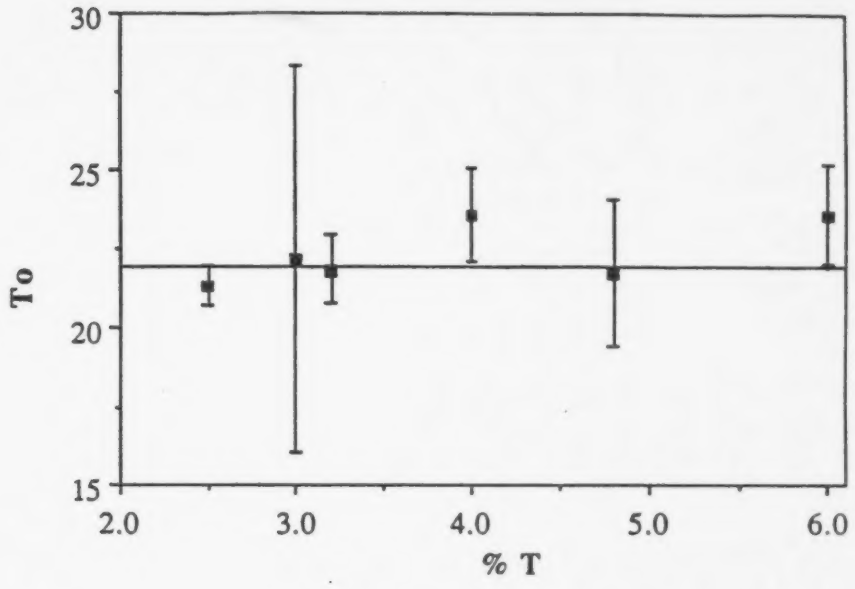


Figure 2.7 Retention time for a vanishingly small DNA fragment as a function of total acrylamide concentration. Data shown at the 95% confidence interval.

sequencing rates reported in the literature for both capillary and slab gel electrophoresis (2,11).

Stegemann *et al.* (2) reported a sequencing rate of 1000 bases/h for a slab gel separation at an electric field strength of 80 V cm^{-1}. Their data appears to have been generated at 50°C while our data were taken at room temperature, 20°C. The difference in electric field strength necessary to obtain this sequencing rate is due to the 2.3%°C^{-1} change in mobility with temperature (12)

The retention time for a vanishingly small DNA fragment, T_0, is independent of %T (Figure 2.7); that is, the retention time of a hypothetical 0-base fragment does not change with %T. This independence of migration rate is not surprising because the 0-base fragment would experience no retardation by the gel. Similar data are reported by Kambara *et al.* (12) for separations of single-strand DNA performed on slab gels at an electric field of 50 V cm^{-1}. The weighted average t_0 is 21.9 ±0.2 min, shown as the horizontal line in the figure. Again, the data are shown at the 95% confidence interval. One datum was Q-tested at the 90% confidence interval from the 6% gel data set.

Combining the results from Figures 2.5 and 2.7, the retention time for a DNA fragment in a 5%C gel at 150 V cm^{-1} in a 35 cm long capillary at room temperature can be written as

$$\text{retention time} = 21.9 \text{ min} + \%T \cdot 0.083 \text{ min base}^{-1} \%T^{-1} \qquad (2\text{-}2)$$

for fragments ranging from 25 to 250 bases in length, gels ranging from 2.5 to 6%T, and room temperature operation. This formula was generated from over 35 electropherograms and represents over 80 h of instrument time.

2.3.3 Electrophoretic Mobility

The relationship presented in equation 2-2 is quite robust in our laboratory. The equation is used to predict the electrophoretic mobility of a DNA sequencing fragment as a function of gel composition and nucleotide size. The electrophoretic mobility, μ, of a fragment of size M is given by

$$\mu_M = \frac{L/E}{\text{retention time}} \qquad (2\text{-}3)$$

where L is the length of the capillary and E is the electric field; the ratio L/E = 0.233 $cm^2\ V^{-1}$ for our experimental conditions. The retention time relationship of equation 2-2 is substituted into equation 2-3 to predict the electrophoretic mobility of DNA fragments ranging in size from 25 to 250 bases and for gels ranging from 2.5 to 6%T.

$$\mu_M = \frac{0.233\ cm^2\ V^{-1}}{1300s + M\cdot(-9.8s\ base^{-1} + 5.0s\ base^{-1}\ \%T^{-1})} \qquad (2\text{-}4)$$

Equation 2-4 is a fundamental description of the electrophoretic behaviour of nucleotides in 5%C polyacrylamide gels. This behaviour must be described accurately for any successful theoretical description of DNA sequencing by gel electrophoresis.

Figure 2.8 presents the predicted mobility for DNA fragments ranging from 25 to 200 bases in length and gels ranging from 2.5 to 6%T. The data extrapolate to a mobility of $1.8 \cdot 10^{-4}\ cm^2\ V^{-1}\ s^{-1}$ for a fragment of zero bases, independent of percentage acrylamide. The mobilities that we obtain are approximately a factor of two smaller than that reported by Holmes and Stellewagen (13) for double-stranded DNA separated at an electric field strength of 3.3 $V\ cm^{-1}$ and at room temperature. The results presented here are about 75% of the values reported by Kambara *et al.*

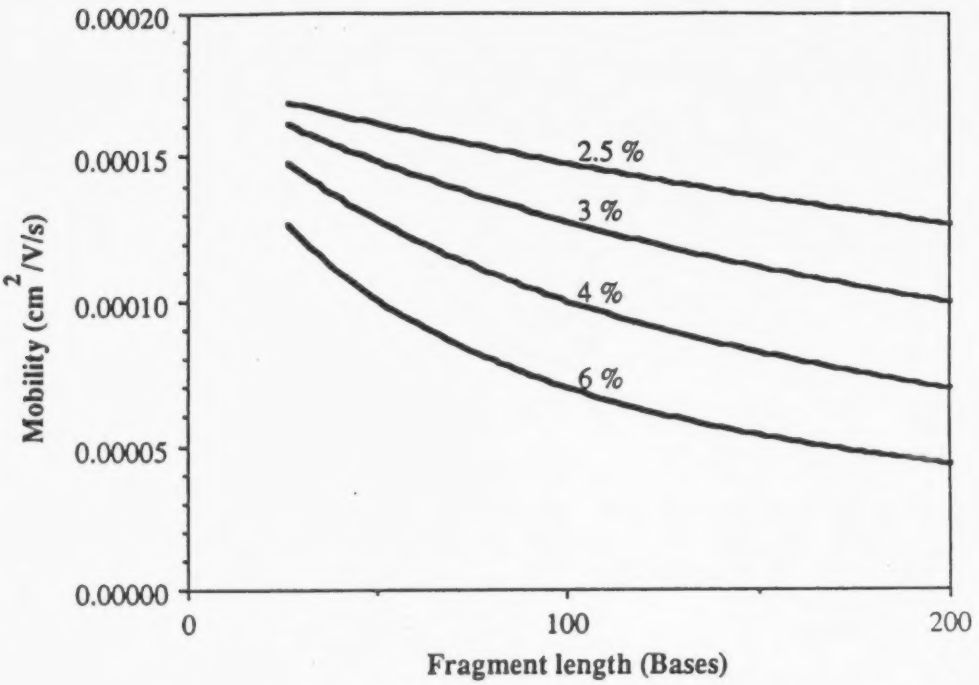


Figure 2.8 Mobility of fluorescently labelled DNA fragments. Total percent acrylamide in the sequencing gel is noted above each curve.

(12) for separation of single-stranded DNA in a slab gel at 48°C. The thermal coefficient of mobility, $2.3\%°C^{-1}$, exactly accounts for the observed difference between the data of Kambara *et al.* (12) and that presented here.

According to the Ogston model (13), mobility may be written as

$$\ln \mu_M = \ln \mu_0 - K_{RM} \cdot \%T \qquad (2\text{-}5)$$

Using equation 2-4 to calculate mobility, Ferguson plots (13) were generated in the range of 2.5 to 6%T for fragments ranging in size from 25 to 100 bases. The Ogston model was fitted to the data. The curves in figure 2.8 extrapolate to a common intercept, $\ln \mu_0 = -8.41 \pm 0.06$, corresponding to a mobility in free solution of $2.2 \pm 0.1 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}. This result is 25% higher than that estimated above from the peak spacing data and one half the value reported for double-stranded DNA (13).

According to the Ogston theory, the retardation of fragments is related to the fractional volume of space in the matrix that is accessible to the analyte (13). To find the %T that produces the same pore size as a particular DNA fragment, the gel composition is found that produces a mobility one-half of that estimated for free solution. Taking the free solution mobility as $2.2 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}, the mobility of interest is $1.1 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}, or $\ln \mu = -9.12$. A 3.5%T gel is estimated to have a pore size of 3.5 nm, equal to the radius of a 100-mer single-stranded fragment; a 4.1%T gel has a pore size of 3.2 nm, equal to the radius of a 75-mer, and a 5.2%T gel has a pore size of 2.8 nm, equal to the radius of a 50-mer. The DNA fragment size is

$$\text{radius} = 0.755(\text{bases})^{1/3} \qquad (2\text{-}6)$$

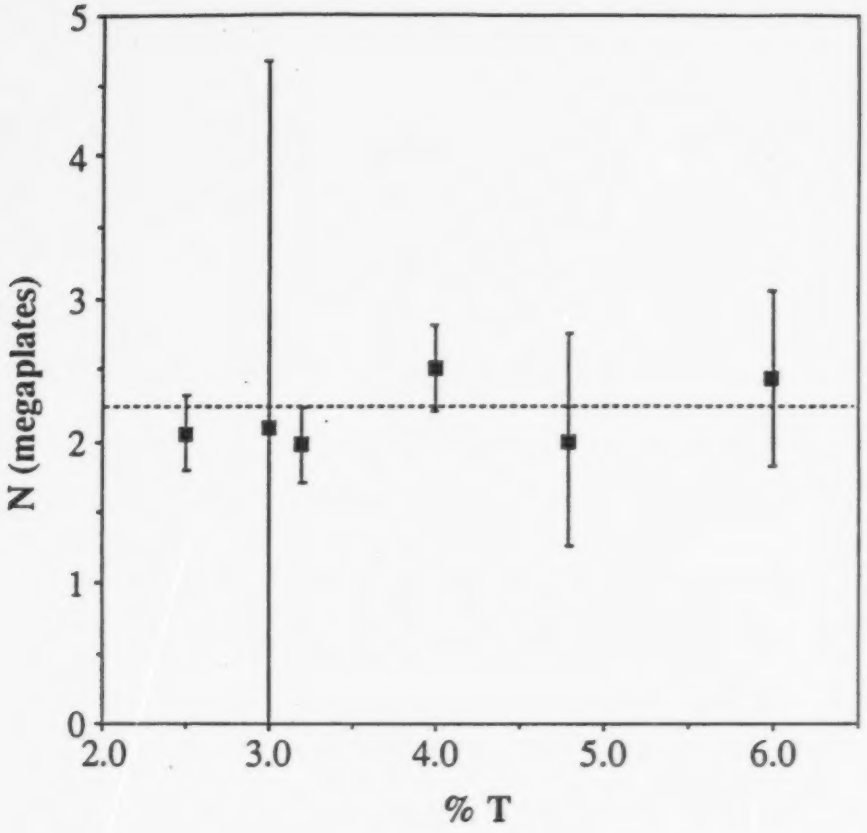


Figure 2.9 Plate count for an 85 base DNA sequencing fragment as a function of total acrylamide concentration. Data are shown at the 95% confidence interval; the dotted line is the weighted average plate count.

and is based on the geometric mean radius for double-stranded DNA (13). A plot of log pore size vs. %T is linear (r=1.000) over the range studied, with slope -0.56. These data are consistent with those observed for Ferguson plots produced on slab gels with double-stranded DNA (13).

2.3.4 Theoretical Plates

The theoretical plate count for base 85 was independent of %T over the range studied, with a weighted average of $2.2\pm0.2\cdot10^6$ plates (Figure 2.9). The data are shown at the 95% confidence interval and the dashed line is the weighted average plate count. Fragments 231 bases in length had a separation efficiency, $N=2.2\pm0.4\cdot10^6$ plates, that also was independent of %T. Plate counts were estimated from the second moment calculation. Slab gel data shows a similar independence of plate count from gel composition (12), although plate count appears to increase slightly with fragment length.

2.3.5 Band Broadening - injection, detection, and thermal gradient

It is interesting to speculate on the origin of the constant plate count. The product of injection voltage and time was varied by several orders of magnitude to determine the effect of column overloading; no improvement in plate count was noted for the smallest sample loadings. Detection time constant and volume do not seem to be important; the system response time would limit plate counts to *ca.* 100 million.

Thermal band broadening could contribute to plate count. Joule heating will produce a parabolic temperature profile in the capillary. For all the gels studied, the

electric current was constant, 1.61±0.01 mA, and independent of gel composition. The heat generated per unit volume in a capillary of radius *r* is

$$Q = \frac{EI}{r^2} = \frac{\lambda C V^2}{L^2} = \frac{150 \text{Vcm}^{-1} \cdot 1.6 \cdot 10^{-6} \text{A}}{\pi (2.5 \cdot 10^{-3} \text{cm})^2} = 1.22 \cdot 10^{1} \text{W cm}^{-3} \qquad (2\text{-}7)$$

Note that the heat dissipated in the capillary scales with voltage squared, at constant molar conductance, λ, and ionic strength, C. Knox (14) has stated that the temperature difference between the axis of the capillary and the inner wall, θ, is given by

$$\theta = \frac{Qr^2}{4\kappa} = \frac{1.22 \cdot 10^{1} \text{W cm}^{-3} \times (2.5 \cdot 10^{-3} \text{cm})^2}{4 \times (4 \cdot 10^{3} \text{Wcm}^{-1}\text{K}^{-1})} = 0.005\text{K} \qquad (2\text{-}8)$$

where κ is the thermal conductivity of the solution, rather arbitrarily estimated as 4 mW $cm^{-1}K^{-1}$ for the 7 M urea, 1X TBE, polyacrylamide solution. The temperature difference across the capillary is very small for 50 μM i.d. capillaries at 200 V cm^{-1} electric fields.

The parabolic temperature profile is translated to a velocity profile by the relative thermal coefficient of mobility, $(d\mu/dT)/\mu = 0.023\ K^{-1}$ (12) (where T= temperature). For the gels used in this study, the relative mobility of a fragment at the centre of the capillary will be 0.00012 (0.012%) higher than the mobility of a fragment at the capillary wall. The maximum theoretical plate count in the presence of this thermally induced velocity profile is related to the diffusion coefficient of the DNA fragment. From the Stokes-Einstein formula, the diffusion coefficient of a molecule is given by (15)

$$D_m = \frac{kT}{6\pi\eta r_{\text{molecular}}} \qquad (2\text{-}9)$$

where k is the Boltzmann constant. The data of Nishikawa and Kambara (1) suggest that the diffusion coefficient of a 100-mer fragment is about $1.0 \cdot 10^{-10}$ cm^2 s^{-1} at 50°C. Converting to 20°C, the diffusion coefficient is expected to be about $9 \cdot 10^{-8}$ cm^2 s^{-1}. Substituting the diffusion coefficient, capillary length, fraction velocity difference, capillary radius, electrophoretic mobility, and electric field strength into Knox's equation

$$N_{thermal} = \frac{24 D_m L}{\left(\frac{d\mu / dT}{\mu} \theta \right)^2 r^2 \mu E}$$

$$= \frac{24 \cdot 9 \cdot 10^{-8} cm^2 s^{-1} \cdot 35 cm}{\left[(0.00012)^2 (2.5 \cdot 10^{-3} cm)^2 (1.0 \cdot 10^{-4} cm^2 V^{-1} s^{-1} \cdot 150 V\ cm^{-1}) \right]} \qquad (2\text{-}10)$$

$$= 5.6 \cdot 10^{10}$$

yields $N_{thermal}$ = 56 billion theoretical plates for an 86-mer fragment. Thermally induced band broadening does not appear to be significant in this capillary system.

$N_{thermal}$ scales inversely with the fifth power of electric field and thermally induced band broadening is insignificant except at very high electric fields in capillaries. For example, an electric field strength of 800 V cm^{-1} will produce a temperature difference of 0.2°C across the 50 μm i.d. capillary, corresponding to $N_{thermal} = 5 \cdot 10^6$ plates. On the other hand, the thermal plate count scales inversely with the sixth power of radius. A 0.5 mm diameter capillary (with similar thermal characteristics as a 0.5 mm thick slab) will have a limiting plate count of 56 000 when operated at 150 V cm^{-1}. Thermal gradients dominate the performance of conventional slab gels at high electric fields.

2.3.6 Band Broadening - longitudinal diffusion

Longitudinal diffusion appears to dominate band broadening in gel filled capillaries. Using the electrophoretic mobility of 85-mer fragments in 4%T gels, the plate count due to longitudinal diffusion is given by

$$N_{\text{longitudinal}} = \frac{\mu V}{2D_m} = \frac{1.0 \cdot 10^{-4}\,\text{cm}^2\text{V}^{-1}\,\text{s}^{-1} \cdot 5250\text{V}}{2 \cdot 9 \cdot 10^{-8}\,\text{cm}^2\,\text{s}^{-1}} = 2.9 \cdot 10^6 \qquad (2\text{-}11)$$

which, given the assumptions in the estimation of diffusion coefficient, is in remarkable agreement with the data. The dependence of plate count on fragment size can be estimated from the mobility formula of equation 2-4, the size dependence of DNA from equation 2-6, and the longitudinal diffusion equation above, and can be written as

$$N_{\text{longitudinal}} = \frac{\mu V}{2D_m} = \frac{0.233\text{V}}{\dfrac{1300\text{s} + \text{M} \cdot \left(-9.8\text{s base}^{-1} + 5.0\text{s base}^{-1} \cdot \%\text{T}\right)}{\dfrac{2\text{kT}}{6\pi\eta 0.755\text{M}^{1/3}}}} = \frac{\text{AM}^{1/3}\text{V}}{1300\text{s} + \text{M} \cdot \left(-9.8\text{s base}^{-1} + 5.0\text{s base}^{-1} \cdot \%\text{T}\right)} \qquad (2\text{-}12)$$

where A is a constant related to temperature and cross-linker concentration. For an 85-mer in a 4%T gel at a voltage of 5250 V, $A \approx 2.7 \cdot 10^5$ s V^{-1}. A plot of expected plate count *vs.* fragment length is shown in Figure 2.10. Plate count maximizes for short fragments, *ca.* 85-mer, but varies by only 25% for fragments ranging in size from 25 to 250 bases. Within experimental error, plate count is independent of fragment length.

Plate count should increase linearly with applied potential. Potential can be increased either by increasing the electric field strength, for a constant length capillary,

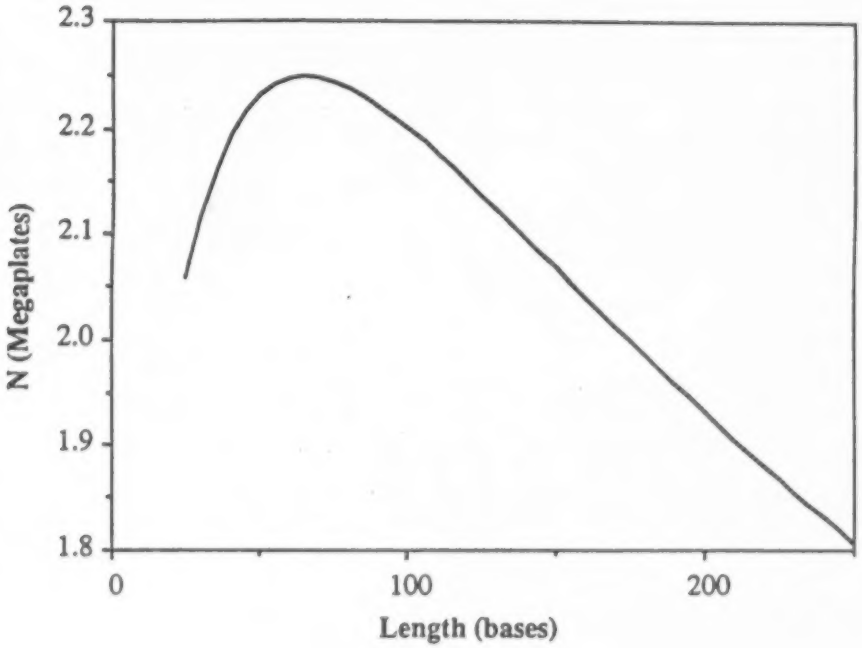


Figure 2.10 Predicted plate count in a 4% total acrylamide gel.

or by increasing the length of the capillary, at constant electric field. The electric field strength cannot be increased without bounds. Polyacrylamide gels are unstable at electric field strengths greater than about 500 V cm^{-1}. Large bubbles form in the capillary at high fields, destroying the separation. However, microbubbles may form at intermediate potentials, creating eddy diffusion and degrading the separation efficiency. It appears that longer capillaries are required to produce very high separation efficiency, albeit at the expense of longer analysis time.

2.3.7 Resolution

Resolution was determined graphically for peaks 85-86 at different %T. The data are shown at the 95% confidence interval in Figure 2.11. The range of resolution observed for the data, from 1 to 2, is quite similar to results from slab gel data (12). Resolution degraded with increased length of sequencing fragment, for all %T studied; a similar phenomenon is present in the data of Kambara *et al.* (12) for fragments ranging in size from 100 to 400 bases.

Resolution of adjacent peaks is related to the theoretical plate count and the relative peak spacing (16,17)

$$\text{resolution} = \frac{\sqrt{N}}{4} \cdot \frac{\text{spacing}}{t_r} \qquad (2\text{-}13)$$

Substituting expressions for plate count, peak spacing, and retention time, the resolution is predicted to be

$$\text{Resolution} = \frac{\sqrt{A \times V \times M^{1/3}} \times \frac{1}{4} \times \left(-9.84\text{sec base}^{-1} + 4.99\text{sec base}^{-1} \times \%T\right)}{\left\{1310\,\text{sec} + M \times \left(-9.84\text{sec base}^{-1} + 4.99\text{sec base}^{-1} \times \%T\right)\right\}^{3/2}} \qquad (2\text{-}14)$$

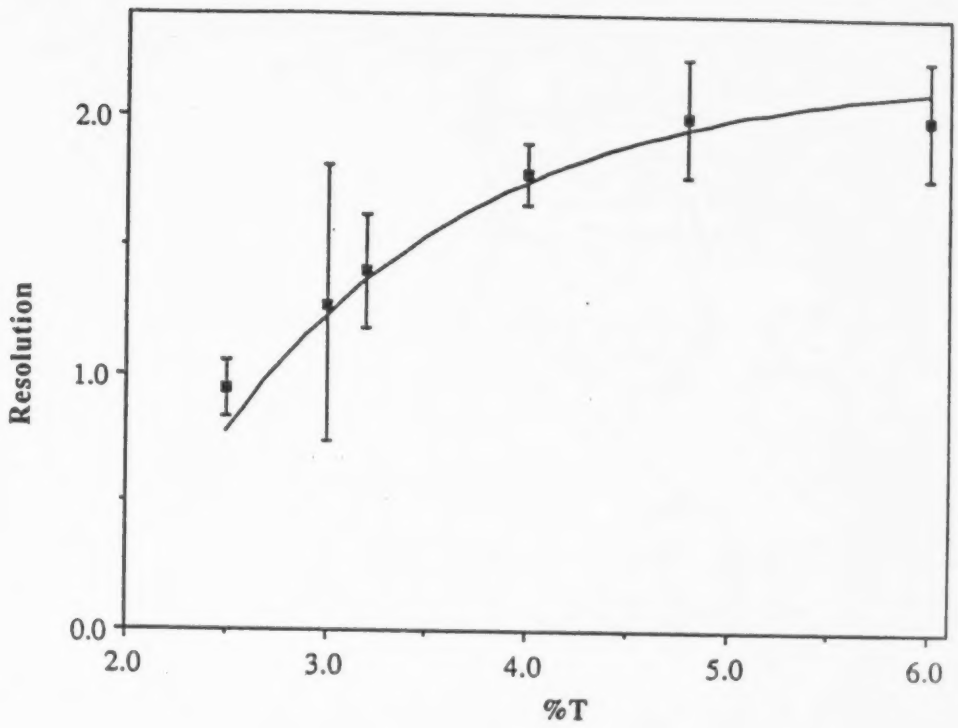


Figure 2.11 Resolution of bases 85-86. Data are shown at the 95% confidence interval. The smooth curve is the prediction of equation 2-14; no parameters were adjusted.

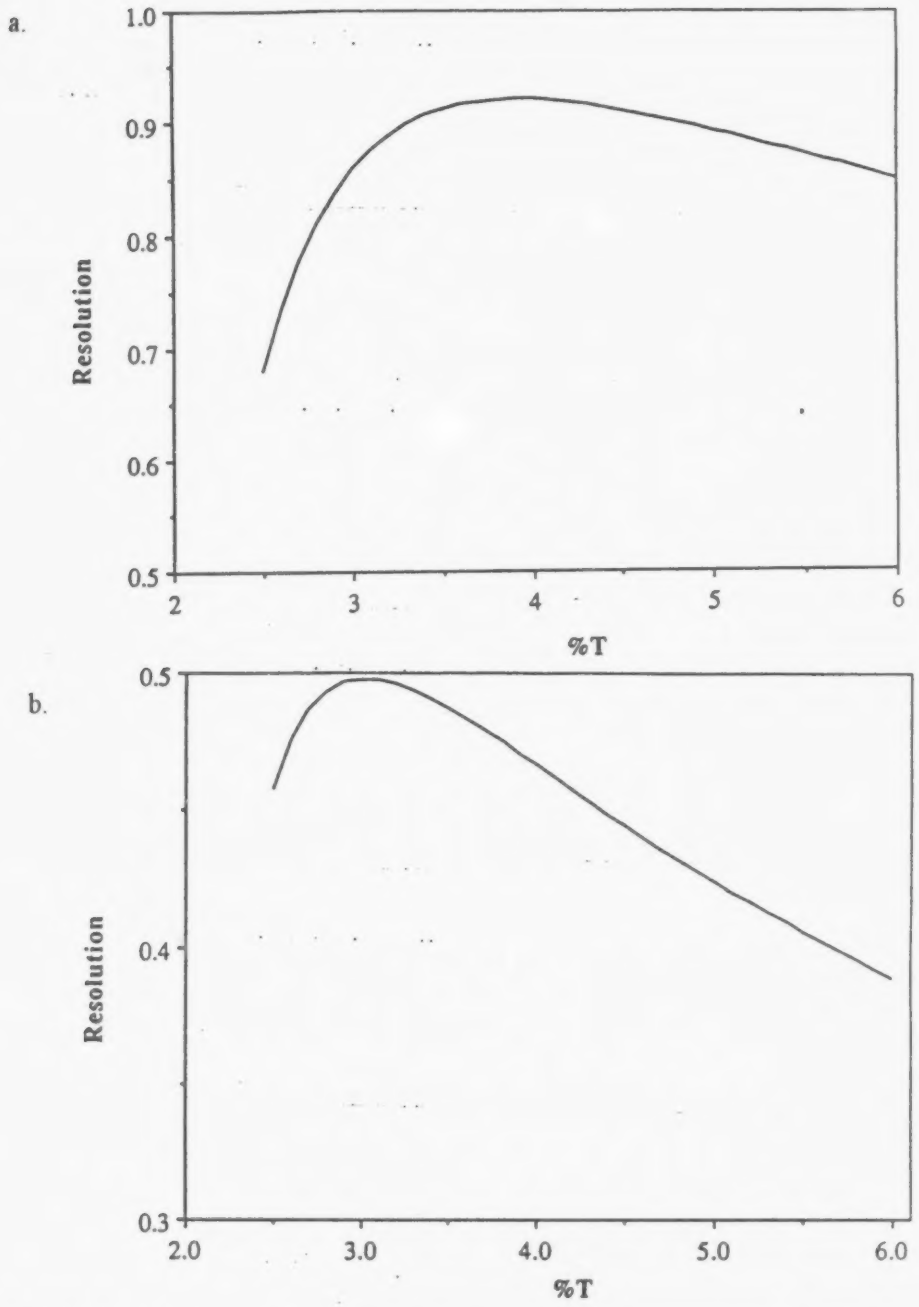


Figure 2.12 Predicted resolution for adjacent DNA fragments as a function of total acrylamide concentration. a. Fragments 250-251 bases in length. b. Fragments 500-501 bases in length.

The smooth curve in Figure 2.11 is a plot of predicted resolution for a fragment 82 bases in length and a potential of 5250 V. Recalling that there are no adjustable parameters in the theory, the agreement with the data is outstanding.

Resolution decreases as *ca.* $M^{-3/2}$ for larger fragments at constant gel composition. For any given length fragment, resolution is maximized for a particular gel composition. Figure 2.12 presents a plot of resolution *vs.* %T for a fragment 250 bases in length. Under the conditions at which the gels are run, the optimum resolution for a 250-mer is produced with a 4%T gel. To analyse long DNA fragments, it is appropriate to use low %T gels.

2.4 Conclusions

This chapter presents phenomenological models for DNA sequencing in polyacrylamide gels with 5% cross-linker concentration. The mobility model has three parameters: fragment length, %T, and the retention time of a vanishingly small DNA fragment. The plate count and resolution model contains one additional parameter, the diffusion coefficient of a DNA fragment of known size in a gel of known concentration.

This model is limited to 5%C polyacrylamide gels ranging from 2.5 to 6%T and operating at 150V cm^{-1} at room temperature. Based on slab-gel data, equation 2-1 requires a quadratic term to describe sequencing data for gel compositions extending to 10%T. Because mobility increases by 2.3% per degree temperature rise, faster separations are expected at higher temperatures. Similarly, different cross-linker concentration or composition will lead to different sequencing rate.

The phenomenological model presented in this chapter is limited to short fragments at an electric field strength of 150 V cm^{-1}. The model is expected to fail for

longer fragments and higher electric fields. Theory for double stranded DNA states that short fragments at low electric field exist in a random coil configuration (18); the mobility of these fragments scales inversely with size. At higher electric fields, or for longer fragments, there is a transition to a stretched or linear configuration; the migration of these fragments is independent of size. Of course, it is not possible to obtain sequence information from fragments in the linear configuration. For double stranded DNA, the transition from random coil to stretched rod configuration is predicted to scale as M/E^2 (18). Extrapolation of the model to fragments longer than 250 bases or for electric fields higher than 150 V cm^{-1} is not warranted.

References

1. Nishikawa, T., Kambara, H., *Electrophoresis*, **12** (1991), 623.
2. Stegemann, J., Schwager, C., Erfle, H., Hewitt, N., Voss, H., Zimmermann, J., Ansorge, W., *Nucleic Acids Res.*, **19** (1991), 675.
3. Kostichka, A.J., Marchbanks, M.K., Brumley, R.L., Drossman, H., Smith, L.M., *Bio/Technology*, **10** (1992), 78.
4. Swerdlow, H., Gesteland, R., *Nucleic Acids Res.*, **18** (1990), 1415.
5. Drossman, H., Luckey, J.A., Kosichka, A.J., D'Cunha, J., Smith, L.M., *Anal. Chem.*, **62** (1990), 900.
6. Cohen, A.S., Najarian, D.R., Karger, B.L., *J. Chromatogr.*, **516** (1990), 49.
7. Swerdlow, H., Wu, S., Harke, H., Dovichi, N.J., *J. Chromatogr.*, **516** (1990), 61.
8. Luckey, J.A., Drossman, H., Kostichka, A.J., Mead, D.A., D'Cunha, J., Norris, T.B., Smith, L.M., *Nucleic Acids Res.*, **18** (1990), 4417.
9. Chen, D.Y., Swerdlow, H.P., Harke, H.R., Zhang, J.Z., Dovichi, N.J., *J. Chromatogr.*, **557** (1991), 237.
10. Karger, A.E., Harris, J.M., Gesteland, R.M., *Nucleic Acids Res.*, **19** (1991), 4955.
11. Swerdlow, H., Zhang, J.Z., Chen, D.Y., Harke, H.R., Grey, R., Wu, S., Dovichi, N.J., Fuller, C., *Anal. Chem.*, **62** (1991), 2835.
12. Kambara, H., Nishikawa, T., Katayama, Y., Yamaguchi, T., *Bio/Technology*, **6** (1988), 816.
13. Holmes, D.L., Stellewagen, N.C., *Electrophoresis*, **12** (1991), 253.
14. Knox, J.H., *Chromatographia*, **26** (1989), 329.

15. Mosher, R.A., Dewey, D., Thormann, W., Saville, D.A., Bier, M., *Anal. Chem.*, **61** (1989), 362.

16. Jorgenson, J.W., Lukacs, K.D., *Anal. Chem.*, **53** (1981), 1298.

17 Giddings, J.C., *Sep. Sci.*, **4** (1969), 181.

18 Noolandi, J., *Can. J. Phys.*, **68** (1990), 1055.

CHAPTER 3

Two-Label Peak-Height Encoded DNA Sequencing by Capillary Gel Electrophoresis[2]

3.1 Introduction

In 1989 Tabor and Richardson reported the effect of manganese on the incorporation of dideoxynucleoside triphosphates (ddNTPs) by T7 DNA polymerase (1). Manganese increases the incorporation rate of the ddNTPs and also produces uniform termination of the DNA sequencing reactions. In 1990, both Ansorge *et al.* and Tabor and Richardson independently reported DNA sequencing protocols based on T7 polymerase with manganese (2-3). Both protocols require a single sequencing reaction with one fluorescently labelled primer. Adjustment of the ddNTP concentrations produces peak heights in a ratio of 8:4:2:1 that encode the DNA sequence. Ansorge also reported a variation of this method in which two sequencing reactions are carried out, each with the same fluorescently labelled primer: ddCTP and ddTTP are present in the first in a ratio of 2:1 while ddATP and ddGTP are present in the second again in a 2:1 ratio. The products of these reactions are separated on adjacent lanes of a polyacrylamide slab gel.

This single reaction technique offers advantages for primer walking applications. Each sample requires only one labelled primer and one reaction. In addition, the separation of the reaction products is carried out in a single lane; this increases sample throughput. Because only a single lane is required the technique is also easily adapted to capillary gel electrophoresis, which provides more rapid separation of the sequencing fragments (4-12).

---

[2]A version of this chapter has been published in *Nucleic Acids Research*, **20** (1992), 4873-4880.

The 8:4:2:1 peak height ratio leads to poor accuracy, typically 90% for fragments shorter than 250 bases (11). There are three sources of error in sequence determination by this technique. First, for each ddNTP the relative variation in peak heights can be about 25% (2). This distribution in peak amplitude leads to errors in identification of the terminal nucleotide, particularly if the peak height ratio is not carefully adjusted. Second, the smallest peaks are frequently lost when sandwiched between peaks of larger amplitude. This problem is more severe for longer fragments where the electrophoretic resolution degrades. For this reason it is difficult to sequence past 250 bases with the four peak height sequencing technique. Third, systematic errors occur due to ghost peaks that are associated with false priming, finite processivity of the polymerase, and contaminant oligonucleotides present in the sample. The ghost peaks, superimposed on the sample peaks, cause changes in the peak heights and errors in identifying the sequence. This effect is most pronounced for the lower amplitude peaks, whose height may be increased by 50 to 100% by the presence of a ghost peak. False priming and artifacts from contaminant oligonucleotides require that highly purified DNA be used for sequence determination.

This chapter describes a modification to this technique. The variation on this method described by Ansorge *et al.* (2) uses two reactions, each with two ddNTPs added in a ratio of 2:1. While it is difficult to control a peak height ratio of 8:4:2:1, a ratio of 2:1 is simple to maintain for any two ddNTPs. Two sequencing reactions are performed, each with a different fluorescently labelled primer and two ddNTPs. The reaction products are pooled and separated on a single gel filled capillary. This method combines the convenience of a single-column separation with the accuracy inherent in the two level discrimination, and the accuracy of multiple labels, at the expense of performing two sequencing reactions.

3.2 Experimental

3.2.1 Electrophoresis

The capillary electrophoresis system is described in detail in section 2.2.1. The capillaries are 50 μm i.d., 190 μm o.d., typically 35 to 40 cm long. The gels are prepared in 5 ml aliquots from carefully degassed solutions of acrylamide and bisacrylamide (4%T, 5%C), 1X TBE and 7 M urea. Polymerisation is initiated by addition of 2 ml of TEMED and 20 ml of 10% (w/v) ammonium persulphate. The gel solution is injected into the capillary with a modified syringe. The gel is covalently bound to the last 2 cm of the capillary with γ-methacryloxypropyltrimethoxysilane to prevent deformation of the gel into the detection cuvette. Polymerisation appears complete in 30 minutes, but capillaries are typically stored overnight before use.

3.2.2 Detector

The detector was initially constructed for the DuPont Genesis sequencing system (Figure 3.1) (11). Fluorescence is excited by a 30 mW argon ion laser operating at 488 nm, and is collected at 90 degrees by a 0.60 NA, 32X microscope objective (Leitz/Wild model 2569-1130). The fluorescence is imaged onto a 0.75 mm diameter pinhole. A dichroic filter splits the fluorescence into two spectral channels. The fluorescence transmitted by the dichroic filter passes through a bandpass interference filter with a 35 nm bandwidth centered at 550 nm, and detected with an R1477 photomultipler tube (PMT). The fluorescence reflected from the dichroic filter passes through a second bandpass inteference filter, centered at 515 nm and with a 25 nm bandwidth, and is detected with an R1477 PMT. The two bandpass filters

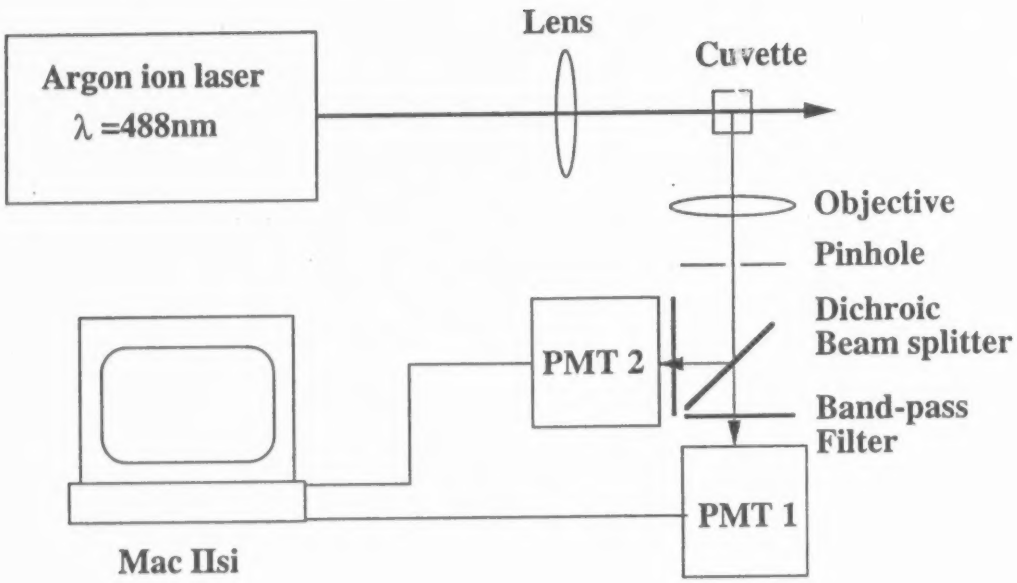


Figure 3.1 Diagram of the two-channel fluorescence detector.

replace the single bandpass filter in the original detector design (Sec. 2.2.1). The output from each PMT is conditioned with a simple low-pass electronic filter with a 0.5 sec time constant and digitised by a National Instruments A/D board in a Macintosh IIsi computer. The data are treated with a binomial filter before presentation. The sheath flow is provided by a syringe pump operating at a flow rate of 0.08 ml/h.

3.2.3 Sample Preparation

Three micrograms of template DNA was added to 2.4 pmol of FAM labelled primer (ABI -21 M13 primer), 2.5 µl 10X MOPS buffer (400 mM MOPS, pH 7.5, 500 mM NaCl, 100 mM $MgCl_2$), 2.5 µl 10X Mn solution (50 mM $MnCl_2$, 150 mM sodium isocitrate) and water to a volume of 18 µl. The primer and template were annealed by heating the mixture to 65 C for 2 minutes, followed by slow cooling to room temperature. Four microlitres of the combined T/G termination mix (the T and G termination mixes were mixed in either a 2:1 or a 3:1 ratio T:G; each mix was 1 mM each dATP, dCTP, dGTP, dTTP, and 3.3 mM of the appropriate ddNTP) was added. The mixture was warmed to 37 C for 2 minutes, then 6 units of Sequenase Version 2.0 and 0.006 units of pyrophosphatase were added and the mixture was incubated at 37 C for 10 minutes The reaction was stopped by addition of 12 µl stop/salt solution (20 mM EDTA, 1 M sodium acetate, pH 8.0) and the DNA was precipitated with 120 µl 98% ethanol. The sample was kept at -20 C for at least 30 minutes, washed with 300 µl ice cold 80% ethanol and dried under vacuum. Identical experimental conditions were used with a JOE labelled primer to yield a nominal peak height ratio of either 2:1 or 3:1 for A and C. The samples were resuspended in 3 ml of a 49:1 mixture of formamide-0.5 M EDTA and the two samples were mixed. The combined

sample was heated to 95 C for two minutes, and injected onto the capillary by applying a 200 V/cm electric field for 20 to 60 seconds.

3.2.4 Sequence Determination

The sequence was interpreted by eye from the smoothed electrophoresis data. The data were plotted in 20 minute intervals with approximately 60 peaks per plot. Two lines were drawn across the plot corresponding to the discrimination level for each fluorescent dye. For fragments between 350 and 400 bases in length, small amplitude fragments occasionally suffer from overlap with neighbouring peaks. All electropherograms were smoothed with a 2 pass binomial filter before presentation.

3.3 Results and discussion

The instrument described here was originally designed for use with the DuPont fluorescently labelled dideoxynucleosides(11). The DuPont system, which is no longer manufactured, excited fluorescence with a single argon ion laser beam and in our version, a single microscope objective collected fluorescence. A simple spectrophotometer was constructed from a dichroic filter that reflects light of wavelengths shorter than 530 nm and transmits longer wavelengths. Spectral bandpass filters isolate fluorescence in the 515 nm and 550 nm regions. Two fluorescently labelled primers (FAM and JOE from Applied Biosystems) are available that match the spectral filters used in the sequencer. The use of two separate bandpass filters provides better spectral resolution than the single bandpass filter used in the earlier version of the instrument (sec. 2.2.1). There is still some optical cross-talk; the FAM labelled primer gives signal in both channels.

The excitation laser was operated at a power of 30 mW. It was determined that the FAM labelled primer was photobleached at laser powers greater than 10 mW. There was however no reduction in signal-to-noise ratio for a laser power of 30 mW so the quality of the FAM signal was not compromised at the higher power. The maximum signal and signal to noise ratio for the JOE labelled primer was obtained at 30 mW laser power. Since JOE generally gave less signal than FAM, and since the length of sequence data, as well as the accuracy, were dependent on signal to noise ratio late in the run, 30 mW was chosen as the optimum laser power for sequencing experiments with this primer pair.

The FAM and JOE labelled primers have been used in the modified peak amplitude sequencing technique. Figures 3.2 and 3.3 show preliminary results for MMTV samples. Figure 3.2a shows the electropherogram of the FAM labelled A and C terminations; FAM gives signal in both channels but only the transmitted channel is plotted. The large, wide peak at about 30 minutes is the excess labelled primer. The DNA sequencing peaks which follow the primer decrease exponentially in intensity as the fragments become longer. The individual peaks that form the exponential envelope have uniform intensities due to the presence of manganese in the sequencing buffer (1). There are two sets of peaks in figure 3.2a. The peaks of highest intensity correspond to the A terminated DNA fragments. A second set of less intense peaks, also with uniformly decreasing intensities, are due to the C terminated DNA fragments. The peak height ratio is 2:1 for A:C.

An expansion of the electropherogram is shown in figure 3.2b. The pattern of peaks was correlated with the known sequence for MMTV for fragments 5 to 320 nucleotides longer than the primer. Some background peaks are present in this sample, for example in the region just before the A peak at 40 minutes. These ghost peaks do not interfere with the identification of the sequence as long as they are less

than 25% the intensity of the smaller peaks. The top panel of figure 3.2c shows fragments 40 to 50 nucleotides longer than the primer. The resolution of the two A peaks 41 and 42 centred at 42.5 minutes is 1.67. The bottom panel shows fragments 200 to 250. The resolution of the A peaks 200 and 201 is 0.90. There are no ambiguities in the peak height encoding. However, identification of the small peaks which code for C terminated fragments becomes difficult as the signal-to-noise ratio decreases late in the run.

Figure 3.3 shows the JOE labelled T and G terminations. The peak height ratio is 2:1 T:G; the large peaks are T's and the small peaks are G's. The pattern of peaks was correlated with the known sequence from 5 to 200 nucleotides. Figure 3.3c shows an expansion of the regions of the electropherograms for fragments 20 to 30 and 110 to 120 nucleotides in length. The difference in peak heights between T 115 and G 116 is less than the theoretical 2:1. This would make discrimination between T and G in an unknown sample difficult. Also present in this panel are two ghost peaks of significant intensity at positions 117 and 119. These could be mistaken for G's in this electropherogram. The known sequence for MMTV shows that there are C's in positions 117 and 119.

A sequencing electropherogram of MMTV DNA is presented in figure 3.4; the peak height ratio is 2:1. Peaks in both traces are A and C; the large peaks are A, the small peaks are C. Peaks in the solid trace only are T and G; large peaks are T and small peaks are G. The sequence was called to 330 nucleotides. The agreement of the called sequence with the known sequence of MMTV was 98%. Four of the seven errors were misidentification of a T as a G due to ambiguous peak heights. Two were misidentification of a C as a G due to poor signal to noise ratio in the reflected channel obscuring the low intensity C peaks.

Figure 3.2a Electropherogram of an A and C terminated MMTV sample. The large peaks are A's, the small peaks are C's.